RUMINAL METABOLISM OF ADDITIVE TREATED SILAGE

by

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I hereby declare that the work for this thesis was carried out solely by myself, except where acknowledgement is made and that the results presented are my own, except the silage analysis, and intake and NH_3-N figures for the in vivo experiments.

This thesis is dedicated to
my mother,
Barbara Ewart (neé Keir),
who was more than dedicated to
me for 27 years,

May I be, someday,
What she saw in me.

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ABSTRACT

Experiments were carried out to investigate the effects of silage additives on ruminal metabolism. Three experimental approaches were adopted; 1) in vivo experiments using sheep; 2) in vitro experiments, and 3) infusion experiments in which silage additives were directly infused into in vitro cultures.

In the first two experiments eleven grass silages were used including materials treated with the additives formic acid, acetic acid and formaldehyde, and wilted silages. Both additive treatment and wilting restricted fermentation and resulted in high residual concentrations of water soluble carbohydrates and low concentrations of protein degradation products.

The in vivo experiments showed that in all cases restriction of in-silo fermentation increased silage voluntary dry matter intake. Microbiological analysis of rumen contents indicated that silage composition influenced the microbial population of the rumen. Numbers of proteolytic organisms were positively correlated with ruminal ammonia concentration and were depressed when formaldehyde-treated silages were ingested. Silage intake influenced ruminal microbial counts, particularly those of cellulolytic organisms.

Development of an in vitro apparatus (The Rumenstat) was an important part of this work. The literature relating to in vitro techniques was reviewed and an apparatus for continuous culture at steady-state was devised, employing established principles and a number of innovations. With the in vitro apparatus, cultures were maintained using the eleven experimental silages as substrates.

The results with these cultures were in accord with those in vivo and confirmed that ruminal characteristics were influenced by compositional differences between the experimental silages, and not by the presence of additives as such.

The effects of silage additives per se were investigated in the third series of experiments. In vitro cultures were infused with increasing concentrations of formic acid, acetic acid, propionic acid and formaldehyde. The organic acids depressed energy metabolism only at concentrations higher than would result from the ingestion of additive-treated silages. Formaldehyde caused a general reduction in ruminal fermentation at low concentrations. The implications of this work to the future development of in vitro rumen technology and the additive-treatment of silage are discussed.

GLOSSARY

μ - microbial growth rate

BC - buffering capacity

C_c - concentration meaured in culture

C_d - concentration measured in dialysate

C_v - additive (infused) concentration in culture vessel

C_{add} - concentration of infusate

D - culture dilution rate (h^{-1})

D_{fac} - multiplication factor applied to dialysate concentrations $= \frac{F_d}{F}$

DM - dry matter

DMI - dry matter intake

F - overall flow rate of culture ($ml.h^{-1}$)

F_a - antifoam flow rate ($ml.h^{-1}$)

F_b - buffer flow rate ($ml.h^{-1}$)

F_d - dialysate flow rate ($ml.h^{-1}$)

F_f / F_s - feed/substrate flow rate ($ml.h^{-1}$)

F_g - gas flow rate ($ml.min^{-1}$)

F_s / F_f - feed/substrate flow rate ($ml.h^{-1}$)

F_{add} - infusate flow rate ($ml.h^{-1}$)

F_{fac} - multiplication factor applied to culture vessel concentrate $= \frac{F}{F_f}$

h - hour

LCD - lowest common denominator

NPN - non protein nitrogen

T_r - turnover rate (d^{-1})

T_t - turnover time (h)

TN - total nitrogen

TSN - total soluble nitrogen

TVFA - total volatile fatty acids

V - volume of culture vessel (ml)

V_a - total volume of antifoam delivered (ml)

V_b - total volume of buffer delivered (ml)

V_d - total volume of dialysate delivered (ml)

V_s - total volume of substrate/feed delivered (ml)

V_t - total volume delivered (ml)

V_{add} - total volume of infusate delivered (ml)

VFA - volatile fatty acids

VN - volatile nitrogen

WSC - water soluble carbohydrate

a.c. - alternating current

J - joule

o.d. - overall diameter

swg - standard wire gauge

V - volt

W - watt

LITERATURE REVIEW

A. SILAGE

INTRODUCTION

The seasonal growth of plants in the UK makes it necessary to conserve a proportion of the plant material, produced in the growing season, for winter feeding. Silage, the material produced by controlled fermentation of a crop (usually grass) of high moisture content, is one means of meeting this need. Control of the fermentation may be by encouragement of anaerobic lactic acid bacteria, or by the direct addition of acids or preservatives.

Traditional or "conventional" silage making is based on a natural fermentation process which depends for its success on the early achievement of anaerobiosis within the silo. Access of oxygen to the herbage leads to aerobic microbial spoilage. If anaerobiosis is achieved and maintained, the sole threat to conservation is clostridial growth which results in the production of carbon dioxide, ammonia and nitrogenous compounds such as amines. Clostridial fermentation is inhibited by low pH, brought about by the lactic acid fermentation, and low water activity, which may be achieved by wilting the crop. As moisture content falls, the value at which pH is limiting rises (Wieringa 1958).

The preparation of natural silage commences with filling the silo. "Silo" is a generic term for a wide variety of equipment from a "clamp" covered and sealed with

plastic sheeting to a substantial metal vessel with mechanical handling equipment. The essential features of a silo are that it must provide for compaction of the herbage and effective sealing. The crop ensiled may be freshly cut or wilted and normally would be harvested at a time when the crop is nutritous and digestible.

Plant juices are released during harvesting as a result of damage to the crop and lactic acid bacteria, though not a substantial part of the indigenous plant flora (Stirling and Whittenbury 1963), proliferate as the silo is loaded (Henderson, McDonald and Woolford 1972). The plant flora are mostly aerobic organisms with little fermentative ability and Bacillus spp., found commonly on herbage, cannot be detected a few hours after ensiling (Langston, Bouma and Conner 1962). The sequence of events during the initial period of ensiling are well documented in reviews by Stirling (1953), Watson and Nash (1960), Whittenbury, McDonald and Bryan-Jones (1967), Whittenbury (1968), Ohyama (1971) and Zimmer (1971).

THE LACTIC ACID FERMENTATION

After ensiling, fermentation proceeds at the expense of sugars, mainly glucose, fructose and sucrose in the case of grass silage, and may follow two general pathways. The pathways are characteristic of two fermentative types of lactic acid bacterium, hetero-fermentative and homofermentative. These pathways are summarised in Table 1.

Table 1. Homo- and heterolactic fermentations.

Homofermentative

(i) 1 Glucose → 2 Lactic acid

(ii) 1 Fructose → 2 Lactic acid

(iii) 1 Pentose → 1 Lactic acid + 1 Acetic acid

Heterofermentative

(i) 1 Glucose → 1 Lactic acid + 1 Ethanol + 1 CO_2 (if fermented)

(ii) 3 Fructose → 1 Lactic acid + 2 Mannitol + 1 Acetic acid + 1 CO_2

(iii) 2 Fructose + 1 Glucose → 1 Lactic acid + 1 Acetic acid + 1 CO_2 + 2 Mannitol (Lactobacillus brevis)

(iv) 1 Pentose → 1 Lactic acid + 1 Acetic acid

Fermentations of the homofermentative type are preferred because heterofermentative activity leads to losses of dry matter (DM) as CO_2, as well as resulting in a larger proportion of neutral products such as mannitol.

Mannitol has also been implicated in the problem of low intake of silage due to palatability effect (Whittenbury 1968) although experimental evidence for this has not been found (McDonald and Henderson 1967).

CLOSTRIDIAL FERMENTATION

Clostridia are virtually the only organisms implicated in the spoilage of anaerobic silage (Gibson 1965) and those fermenting lactate to butyrate are of greatest importance. Lactate-fermenting clostridia have been shown to multiply for a few hours after the silo has sealed, and thereafter persist as spores (Gibson 1965). Whilst such saccharolytic clostridia will also ferment residual sugars (MacGregor and Whittenbury 1967) the significance of their activity lies in the pH reversal brought about by the production of one mole of butyrate from two moles of lactate. This change brings about conditions favourable to the proteolytic clostridia which produce compounds characteristic of putrefaction, from amino acids. (Table 2)

USE OF ADDITIVES IN SILAGE MAKING

a) GENERAL. The purpose of using additives in silage making is to encourage preservation by promoting useful microbial activity (stimulation), or by restraining detrimental activity (inhibition). Whilst complete

Table 2. Fermentation of amino acids by Clostridia.

(i) Coupled oxidation-reduction reactions (Stickland)

1 Alanine + 2 Glycine → 3 Acetic acid + 3 NH_3 + CO_2

(ii) Deamination

3 Alanine → 2 Propionic acid + Acetic acid + 3 NH_3 + CO_2

1 Valine → isobuturic acid + NH_3 + CO_2

1 Leucine → isovaleric acid + NH_3 + CO_2

(iii) Decarboxylation

Histidine → Histamine

Lysine → Cadaverine

Arginine → Ornithine → Putrescine

Tryptophan → Tryptamine

Tyrosine → Tyramine

inhibition of microbial activity would seem to be the surest method of preservation, Whittenbury (1968) suggested two problems which might arise from this approach : 1) A substance which inhibits microbial growth and remains active in the silage could present difficulties at the time of feeding by inhibiting the microflora of the rumen : 2) Substances with a short period of activity or which are ineffective under aerobic conditions will allow rapid deterioration when the silo is unloaded.

Nutritional supplements are sometimes added to silage to improve the nutritional value, without necessarily influencing the fermentation per se. Nitrogen content may be increased by the addition of urea; this has been shown to have no effect on the contents of lactic, acetic and butyric acids (Barbier 1961). Nitrogen and phosphate contents may be increased by addition, to the silage, of diammonium phosphate, without influencing the fermentation (Watson and Nash 1960).

b) STIMULANT ADDITIVES. Direct stimulation of the lactic acid fermentation by the addition of lactic acid bacteria has not proved successful (Watson and Nash 1960), particularly when water soluble carbohydrate (WSC) is high (McDonald, Stirling, Henderson and Whittenbury 1964). Wieringa and Hengeveld (1963) found that, with a crop of low WSC, the addition of a liquid culture of lactic acid bacteria (10 l/tonne) was beneficial, but when WSC was very low ($<$ 6.3% of DM) silage of poor quality was produced, even with an inoculum.

Stimulation of the lactic acid fermentation indirectly, by the addition of carbohydrate-rich substrates such as barley meal, beet pulp, glucose, maize, molasses or sugar, has been attempted . Many of these materials were used on a regular commercial basis for a number of years, but their cost, together with the increasing use of inhibitory additives, has led to a decrease in their popularity. The most widely applied additive of the stimulatory type was molasses although, according to Whittenbury (1968), the high proportion of fructose in this compound encourages the less desirable heterofermentative organisms.

c) INHIBITORY ADDITIVES. Inhibitory additives may be classified into two types, sterilants and acidifying agents.

Sterilants. The sterilants used as silage additives are compounds such as antibiotics, formalin, sodium metabisulphite and SO_2. Antibiotics have always held promise as additives because they are effective in low concentrations and highly specific in action; many have been proposed, including aureomycin, streptomycin, terramycin, oleandomycin and penicillin but, according to Wing and Wilcox (1960), none of these improved silage fermentation quality. The use of therapeutically important antibiotics in agriculture is questionable in any case, because the risk of a drug-resistant population being developed is considerable. The choice of antibiotics available as silage additives is thus limited to the non therapeutic types (Swan 1968).

Brown and Kerr (1965) state that zinc bacitracin

and molasses improved grass silage, although it was not clear from their studies whether the antibiotics or carbohydrate caused the improvement. Bacitracin was found (De Vuyst, Vanbelle, Arnould, Maesmans, Vervack and Moreels 1965) to have a beneficial effect on red clover silage. The streptococcal antibiotic nisin, which, like zinc bacitracin inhibits sporeformers, is ineffective because it is degraded by the silage microflora (Galesloot 1956), it has only limited effect against butyric acid organisms (Frolich 1958) and antagonises the lactic acid bacteria (Flam 1967). None of these considerations, however, prevented Whittenbury (1968) proposing the use of nisin-producing streptococci as a silage additive.

Sodium metabisulphite is a highly water-soluble compound and a powerful reducing agent when moist. Much research has been applied to the investigation of meta-bisulphite as a silage additive and the general conclusion is that, whilst it does not prevent proteolysis in silage, amino acid breakdown is inhibited (De Vuyst, Vervack and Arnould 1968, Durand-Salomon and Zelter 1960, Macpherson, Wylam and Ramstad 1957 and Alderman, Cowan, Bratzler and Swift 1954). SO_2 gas has similar effects as sodium metabisulphite (Skaggs and Knodt 1952, Kroulik, Burkley, Gordon, Wiseman and Melin 1955), but difficulties in application and the evolution of SO_2 on opening the silo reduce its practical usefulness as an additive (Jensen, Mølle, Møller and Pedersen 1962).

Formaldehyde has been used as a decontaminant,

sterilant and biological preservative for over 80 years. Large quantities are currently manufactured for industrial use; the compound is available as paraformaldehyde, a solid polymer, or as formalin a 40% aqueous solution (Hoffman 1971). Early work on formaldehyde as a silage additive has been summarised by Watson and Nash (1960). Apart from some work in the 1930's formaldehyde was not considered as a practical silage additive until 1970 when the experiments of Gordon, Derbyshire, Wiseman, Kane and Melin (1961); Jackson and Forbes (1970) and McLeod, Wilkins and Raymond (1970) suggested that acids produced in fermented silage could be responsible for limiting intake. The desirability of a chemical additive, functional without the presence of acids and inhibiting the lactic acid fermentation, became apparent. McLeod et al. (1970) showed that high levels of formaldehyde treatment (approximately 4g/kg $\simeq$ 2 gallons formalin per ton fresh grass) produced high pH, low volatile fatty acid (VFA) silages with intake levels higher than the corresponding fermented silages.

Apart from being unpleasant to handle, formaldehyde has two potential disadvantages as a silage additive. Firstly, formaldehyde acts as a protein binding agent by forming intermolecular cross-linkages (Hoffman 1971), a consequence of which is reduced digestion of protein in the rumen (Ferguson, Hemsley and Reis 1967) resulting in possible nitrogen starvation of the microflora.

Secondly, formaldehyde treated silages are prone

to aerobic deterioration on unloading. Silages treated at a rate of 3g/kg (fresh grass) were found to sustain mould growth and greater temperature rises on unloading, than untreated material (Wilson, Wilkins and Cook 1971).

Acidifying agents. The earliest acidifying agents were mixtures of mineral acids (for example the A.I.V. process), which were used in Scandinavian countries (Watson and Nash 1960). The A.I.V. process involved reducing the pH of the herbage to a value below 4, usually with a 2N mixture of hydrochloric and sulphuric acids. At this pH fermentation is prevented, amino acid breakdown is reduced and clostridia are inhibited (De Vuyst, Vervack and Arnould 1968). The A.I.V. process was never popular in the U.K. because the acids are difficult to handle and corrosive (Woodman 1949). Organic acids have come into use in recent years and most important among these are the following:

1) Formic acid. This compound has replaced mineral acids in Scandinavia and is currently established as the basis of a commercial product commonly used in this country. This product, ("Add-F"[1]) contains 85% formic acid and application at 2.37 kg/tonne fresh grass is recommended. The present commercial success of formic acid additives contrasts with the disappointing results of earlier experimental work (Watson and Nash 1960). Formic acid is effective as an additive only when the crop is chopped (Saue and Brierem 1969) and the increasing use of this

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[1] "Add-F" marketed by BP Chemicals (International) Ltd.

compound reflects developments in forage harvesting machinery. Aas and Naerland (1966) devised a spray applicator for use during harvesting, which overcame the problem of achieving uniform dispersal of additive in the chopped material.

The effect of formic acid on the fermentation of low DM grass was studied by Henderson and McDonald (1971). The immediate effect of treatments at 2.2, 3.4 and 5.1 kg/tonne fresh grass was a reduction in pH to values of 3.8 to 4.1, and the conservation of substantial proportions of the WSC. Small amounts of lactic acid were formed with the lowest treatment, suggesting that inhibition of the lactic acid bacteria was not complete. As well as containing more ethanol than the control, all the treated silages had greater losses of DM owing to effluent production. With a formic acid addition of 2.3 kg/tonne fresh grass to timothy/ryegrass, Castle and Watson (1970) produced silages of lower pH and higher lactic acid than untreated controls, whereas the same rate of addition to Lolium perenne by Wilkins and Wilson (1968) resulted in silages with pH values identical to the control but lactic acid contents which were lower. Despite the uncertainty regarding the effects of formic acid during ensiling, it does appear that treated silages are nutritionally superior to untreated material from the same crop (Waldo, Smith, Miller and Moore 1969; Castle and Watson 1970), particularly with respect to DM intake.

2) Acetic acid. Acetic acid is being considered as a

silage additive (Frazer 1971) and, as the next member of the homologous series after formic acid, should be a more effective microbial inhibitor (Niemann 1954). Depression of silage intake by large quantities of free acetic acid is considered to be a potential disadvantage which may limit its use. When Hutchinson and Wilkins (1971) used intra-ruminal infusions of acetic acid to investigate its effect, intake was depressed. A similar depression of fresh grass intake by intra-ruminal infusion of acetic acid was obtained by Rook, Balch, Campling and Fisher (1963).

3) Propionic acid. Propionic acid is recognised as a food preservative (Huitson 1968) and is established in the agricultural industry as a preservative of stored cereal grains for animal feed ("Propcorn"[2]). Daniel, Honig, Weise and Zimmer (1970) found that propionic acid gave good results with grass silage reducing the production of lactic acid and fermentation gases, and restricting deamination of amino acids.

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[2] "Propcorn" is manufactured by BP Chemicals (International) Ltd.

B. RUMEN MICROBIOLOGY & BIOCHEMISTRY.

THE RUMEN AS AN ECOSYSTEM.

In physical terms the rumen environment is stable; the pH is generally in the range 5.5 to 6.5 (Smith 1941), temperature remains between 39°C and 40.5°C (Krzywaneck 1929) and regular mixing is provided. These factors, together with the highly reduced chemical conditions which prevail (Broberg 1957, Smith and Hungate 1958), define the main constraints with which any microbe, which is to succeed in the rumen, must contend.

Consumption of forage by the host is not a continuous and regular process and the material consumed may be of irregular quality. The process of rumination imposes a further dynamic influence on the microbial population and contributes to the cycle of events which follows each intake of food (Warner 1956). Selective pressures on the microbes thus change with time as, for example, food material is consumed and waste products and microbial cells accumulate. Organisms with different chracteristics will successively exploit their respective niches when the cycle of events passes through the phase when conditions to which they are best adapted prevail (Hobson 1972). Considerable diversity of microbial species arises as a consequence of this intrinsically variable ecosystem.

BACTERIA OF THE RUMEN.

Bacterial species encountered include both

facultative anaerobes (eg Coliform sp., Propionibacteria sp., Lactobacilli sp., and Streptococci sp.) and obligate anaerobes, including some twenty species of non-sporeforming rods, in addition to sporeforming rods (Clostridium sp.) and cocci (Bryant 1959, Hungate 1966). Total numbers of bacteria are in the order of 10 - 50 x 10^9/ml (Hungate 1966) although under some circumstances numbers can be considerably fewer than this.

Hungate (1966) used the following descriptions to group rumen bacteria :

1. Cellulose digesters.
2. Starch digesters.
3. Hemicellulose digesters.
4. Sugar fermenters.
5. Acid utilisers.
6. Methanogens.
7. Lipolytic bacteria.

Examples of most genera are to be found under each of these headings, which are not exclusive to individual organisms. Table 3 lists the more important rumen bacteria.

PROTOZOA OF THE RUMEN.

Rumen protozoa number some 2 x 10^6/ml (Barnett and Reid 1961) the majority being ciliates though a few species of small flagellates are found, contributing

TABLE 3 BACTERIAL SPECIES COMMON IN THE RUMEN

ORGANISM	MORPHOLOGY	NICHE	PRODUCTS			GROWTH REQUIREMENTS			
			C_2	C_3	C_4	VFA	CO_2	NH_3	AAs
Bacteroides succinogenes	Rod	Cellulose Digestion	+	-	-	E	E	E	-
Ruminococcus albus	Coccus	Fibre Digestion	+	-	-	E	E	E	+
Bacteroides amylophilus	Rod/ Irregular	Starch Digestion	+	-	-	-	E	E	-
Succinomonas amylolytica	Coccoid/ Rod	Starch Digestion	+	+	-	E	E	?	-
Peptostreptococcus elsdenii	Cocci/ Chains	Lactate Fermentation	+	+	+	-	-	-	E
Clostridium locheadii	Rods	Cellulose Digestion	+	-	+	-	-	-	E
Butyrivibro fibrisolvens	Curved Rods	Wide Adaptation	+	-	+	E	±	E	E
Selenomonas ruminantium	Crescent	Wide Adaptation	+	+	±	E	±	-	E
Streptococcus bovis	Cocci	Starch Digestion	±	-	-	±	+	±	E
Eubacterium ruminantum	Coccoid/ Rod	Sugar/Xylan Fermentation	+	-	+	E	?	E	-

PRODUCTS CODE: + = PRODUCED; - = NOT PRODUCED; ± = PRODUCED BY SOME STRAINS

NUTRIENTS CODE: E = ESSENTIAL IN SOME MEDIA; + = STIMULATES GROWTH; - = DOES NOT STIMULATE GROWTH

/ BASED ON HUNGATE (1966) AND BRYANT (1959) /

approximately 2.5×10^3 to the above number. There are at least 30 species of ruminal protozoa capable of a range of biochemical activities similar to those listed above for the rumen bacteria. There are no methanogenic protozoans. Table 4 gives the classification of these organisms together with some example species (Hungate 1966).

Table 4. Rumen Protozoa.

	Holotrichs	Entodiniomorphs (= Oligotrichs)
Subclass	Holotrichia	Spirotrichia
Order	Trichostomatida	Entodiniomorpha
Family	Isotrichidae	Ophryoscolecidae
Genera	Isotricha, Dasytricha	Entodinium, Diplodinum Epidinium, Ophryoscolex
Examples	Isotricha prostoma Dasytricha ruminantum	Diplodinium dertatum Epidinium ecaudatum
Substrates	Sugars	Cellulose / Starch

BIOCHEMISTRY OF THE RUMEN.

The rumen does not have a regulated and fixed biochemistry such as is generally found in mammalian tissues. As already discussed, the microbial population is ecologically unstable, the substrate ingested by the host being the chief factor influencing the microbial and biochemical pattern prevailing (Hungate 1966). On a roughage diet, for example, the cellulose and

hemicellulose digesting organisms will dominate the microflora whereas on a concentrate diet (with a high proportion of starch) amylolytic and sugar-fermenting organisms will thrive (Warner 1965). Different groups of organisms possess different biochemical propensities which give rise to distinctive biochemical patterns in the rumen.

CARBOHYDRATE FERMENTATIONS.

CELLULOSE DIGESTION. Cellulose, a β-D glucose polymer comprising long chain molecules with mol. wt from 200,000 to 1,000,000 (Thimann 1963) is attacked by enzymes produced only by certain micro-organisms. The more important cellulose digesters in the rumen are Bacteroides succinogens, Butyrivibrio fibrisolvens, Clostridium lochheadii, and Ruminococcus albus and certain of the entodiniomorph protozoa (Tables 3 and 4). The presence of sugars inhibits the cellulase enzymes produced by these organisms (or inhibits their production) and sugars do not accumulate in their cultures (Smith and Hungate 1966). Cellulose in the rumen is broken down into the disaccharide cellobiose (Norman and Fuller 1942) and saccharides containing more than two glucose units are not normally found during cellulose breakdown.

STARCH DIGESTION. Many of the cellulolytic organisms of the rumen also attack starch (Bryant 1959) including most strains of the species named above. Many starch

digesting strains are encountered among the non cellulolytic species, for example Stretococcus bovis, Bacteroides amylophilus and Succinimonas amylolytica. Starch is actively ingested by all of the entodiniomorphs (Sudgen 1953) and used as a storage compound by all types of ruminal protozoa (Thomas 1960, Oxford 1959). Bacterial and protozoal attack is rapid and very little starch is passed out of the rumen (Marston 1948).

HEMICELLULOSE DIGESTION. Hemicelluloses are an ill-defined group of compounds which accompany cellulose in plant cell walls and on hydrolysis yield hexoses, pentoses and frequently uronic acids. All cellulolytic organisms attack hemicelluloses, producing mainly arabinose and xylose (Hungate 1957).

SUGAR FERMENTATION. The ability to ferment sugar is possessed by all the polysaccharide-hydrolysing organisms (Hungate 1966). Polysaccharide hydrolysis tends to be inhibited by the presence of sugars, "end product inhibition", depletion of non carbohydrate substrates by saccharoclastic organisms, excess production of acid (also resulting in "end product inhibition") each probably playing a part in this (Hamilton 1942, Hoflund, Quinn and Clark 1948, W. Smith and Hungate in Hungate 1966).

PRODUCTS OF FERMENTATION. Because of the factors discussed in the foregoing, sugars tend to arise in limited concentrations in the rumen and certainly only minute amounts are absorbed through the epithelium (Schambye 1951 [a,b]).

Thus a pool of sugars is constantly replenished from the sources discussed above; this pool is small and subject to extremely rapid turnover. Apart from some interconversions and isomerisation virtually all of the sugar is metabolised by the rumen micro-organisms (Fig, 1) resulting in the release of energy and the production of volatile fatty acids (VFAs) (Gunsalus and Shuster 1961). The unit of energy in biological systems, at the molecular level, is bound in the hydrophosphate group of adenosine triphosphate (ATP), or occasionally in other nucleoside triphosphates (Rose 1968). Since the quantitatively most important foodstuff components of the ruminant diet are the pentose and hexose polymers, they are the greatest source of energy to the rumen organisms (Walker 1965).

There is no direct evidence that hydrolysis of the polymers involves energy-yielding reactions, but it is possible that the splitting of cellobiose may yield a high energy bond without the expenditure of ATP, by means of a pyrophosphate reaction (Ayers 1959, Hungate 1963). There is, however, evidence of a β-glucosidase produced in the rumen which results in unphosphorylated glucose by simple hydrolysis of cellobiose (Conchie 1954). It seems, too, that the maltose dimer produced by the amylolytic breakdown of starch is split by the phosphorylase resulting in half the product being glucose-1-phosphate (Hobson and

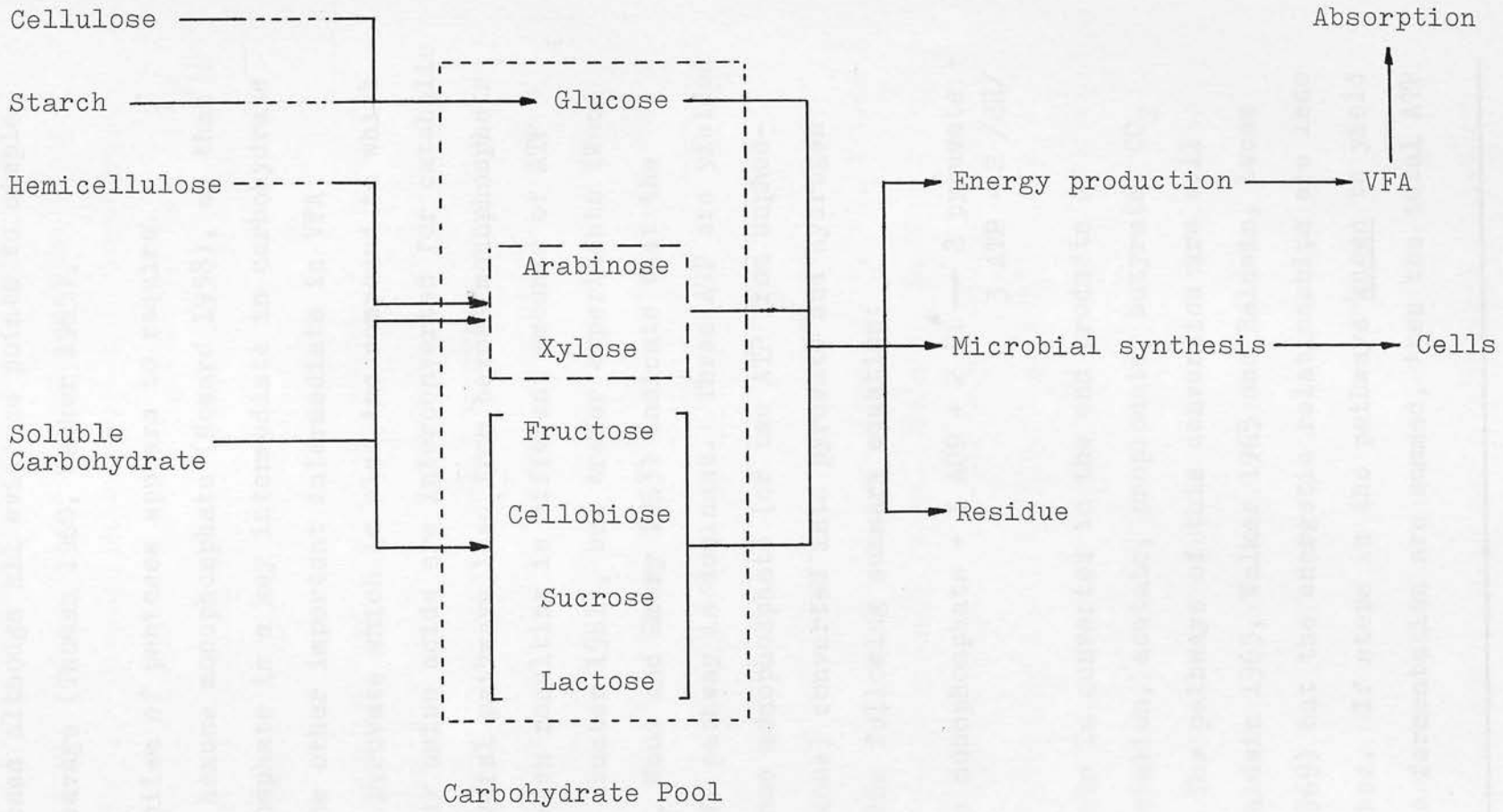

Fig. 1 Overall carbohydrate pathways in the rumen.

McPherson 1952). Little is known about the degradation of hemicelluloses although all evidence points to simple hydrolytic cleavage (Thomas 1960, Walker 1965).

Metabolism of pentoses appears to require conversion to hexose monophosphate (Howard 1958), so that hexose monophosphate is a key intermediate in carbohydrate breakdown. The other important intermediate in VFA production is pyruvate which is also the compound to which lipids and many amino acids are interconverted for catabolic purposes. Several pathways lead from hexose monophosphate to pyruvate, each resulting in different amounts of ATP (Gunsalus and Shuster 1961), but tracer experiments (Wood 1961, Baldwin, Wood and Emery 1963) indicate that the Embden Myerhoff pathway is dominant. Three ATP are yielded from each hexose monophosphate (or two ATP from unphosphorylated hexose) converted into pyruvate and hydrogen according to the following summary equation:

$$\text{Hexose monophosphate} + 3\ \text{ADP} + 2\ \text{Pi}^{*} \longrightarrow 2\ \text{pyruvate} + 3\ \text{ATP} + 2\ /2\text{H}/$$

Pyruvate is converted to the end products of ruminal fermentation, acetate, propionate, butyrate, CO_2 and methane. The pathways of this conversion are well documented (Baldwin 1965, Walker 1965 and Baldwin, Lucas and Cabrera 1969) but the energetic relationships are less well established. If steps in the pathways known to yield ATP during the fermentation are summed, then the total ATP

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* Pi = inorganic phosphate

yield from the fermentation of carbohydrates, when the proportions of VFA, biochemical date and growth data are taken into account, appears inadequate (Baldwin, Lucas and Cabrera 1969). Several steps where ADP phosphorylation may occur have consequently been postulated so that two different overall equations may be presented for some of the pathways known to arise (Table 5).

FORMATION OF ACETATE, PROPIONATE AND BUTYRATE. Pyruvate may be decarboxylated to form acetate by several routes of which two have been demonstrated in rumen bacteria. The clostridial phosphoroclastic reaction wherein acetyl CoA (coenzyme A) is the primary intermediate has been characterised in Cl. butyricum (Mortloch, Valentine and Wolfe 1959) and Peptostreptococcus elsdenii (Peel 1960). The reactions require CoA, Fe^{++} and diphosphothiamine (DPT) as cofactors and produce acetyl phosphate, CO_2 and H_2 according to the scheme in Fig. 2(a) (Wolfe and O'Kane 1955). The second pathway to have been demonstrated in the rumen is the so-called coli-aerogenes phosphoroclastic (or formate phosphoroclastic) type, Fig. 2(b). This system requires CoA, DPT, Fe^{++} and Mn^{++} or Mg^{++} (McCormick, Ordal and Whiteley 1962) and its products are acetyl phosphate and formate. Formate is rapidly converted to CO_2, H_2 and methane in the rumen (Carroll and Hungate 1955) by one, or both, of the systems shown in Fig. 3 (Peck, Smith and Gest 1957, Brill, Wolin and Wolfe 1964). The formate phosphoroclastic pathway is summarised in Fig. 2(b) and, in common with the clostridial phosphoro-

Table 5 Ruminal evolution of VFA, known and postulated overall equations.

Acetate

(1) Pyruvate + NAD + ADP + Pi → Acetate + NADH + CO_2 + ATP

(Clostridial phosphoroclastic)

(2) Pyruvate + ADP + Pi → Acetate + Formate + ATP

(Coli-aerogenes phosphoroclastic)

Propionate

(3) Pyruvate + 2 NADH + ADP + Pi → Propionate + 2 NAD + ATP

(Known + Postulated ADP phosphoroclastic steps:
Direct reductive pathways - acrylate or
methyl malonyl CoA carboxylase)

(4) Pyruvate + 2 NADH → Propionate + 2 NAD

(Dicarboxylic acid pathway)

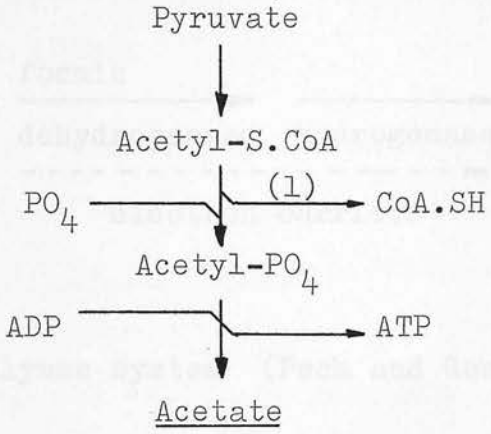
Butyrate

(5) 2 Pyruvate + 2 ADP + 2 Pi → Butyrate + 2 CO_2 + 2 ATP

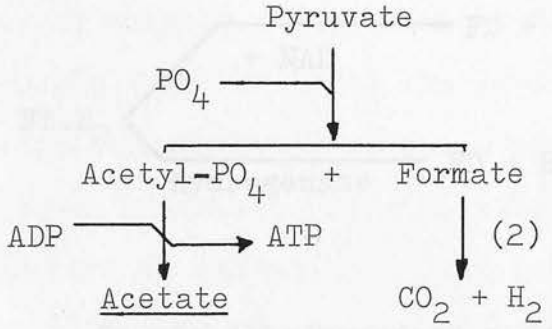
(Known + Postulated phosphorylation steps)

(6) 2 Pyruvate → Butyrate + 2 CO_2

(Known phosphorylation steps only)



a) Clostridial phsophoroclastic pathway.

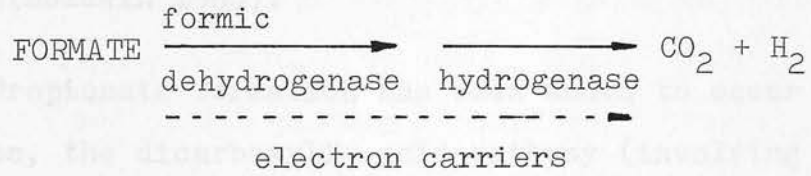


b) Coli-aerogenes phosphoroclastic pathway.

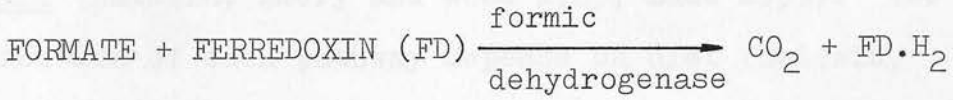
Fig. 2 Pathways of acetate formation.

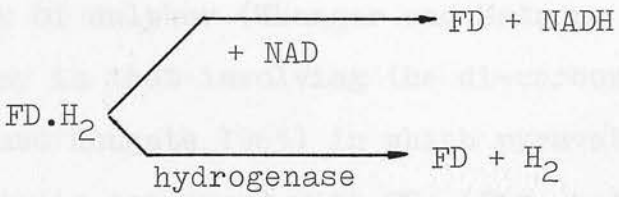
(1) Phosphotransacetylase enzyme.

(2) Formate dehydrogenase enzyme.



Hydrogenlyase system (Peck and Gest 1957)





Formic dehydrogenase : ferredoxin : NAD system (Brill, Wolin and Wolfe 1964)

Fig. 3 Possible mechanisms of formate degradation.

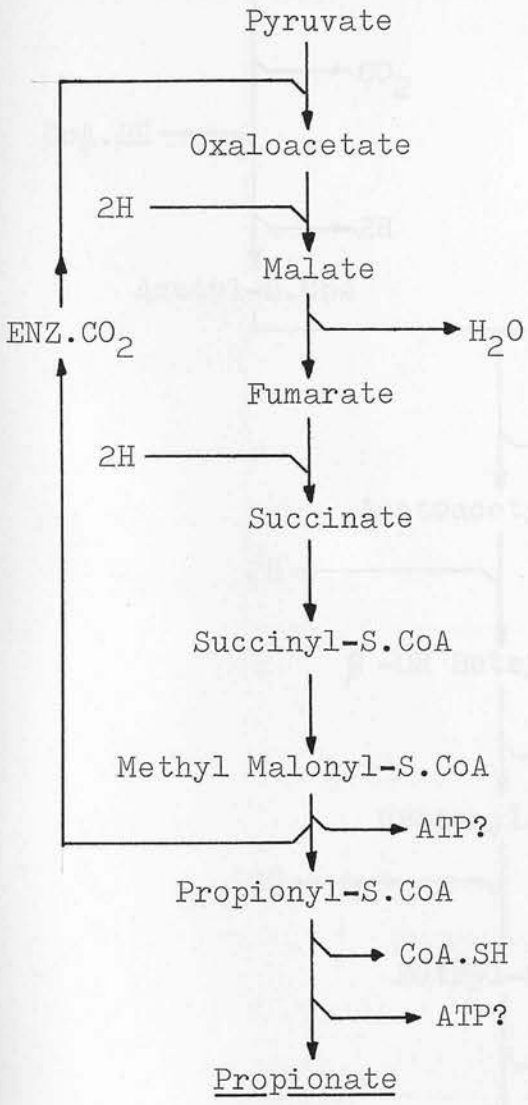
clastic scheme, is known to generate one ATP per pyruvate consumed (Baldwin 1965).

Propionate formation has been shown to occur by two routes, the dicarboxylic acid pathway (involving succinate) found in V. alcalescens (Stadman and Vagelos 1957) and in propionibacterium, and the direct reductive pathway (involving CoA esters of acrylate and lactate) which has been demonstrated in Cl. propionicum and P. elsdenii (Baldwin, Emery and Wood 1965, Ladd 1959). The contribution of each pathway depends on diet (Baldwin, Wood and Emery 1962, 1963) and, in particular, the availability of sulphur (Whanger and Matrone 1967). The major pathway is that involving the di-carboxylic acids (Blackburn and Hungate 1963) in which pyruvate or phospho-enol-pyruvate is condensed with CO_2 (Fig. 4a), to form oxaloacetate (or, if the malic enzyme is involved, malate). Oxaloacetate is reduced to malate by malic dehydrogenase or a lactate-malate transhydrogenase (Dolin, Phares and Long 1964). The malate is dehydrated to fumarate which, in turn, is reduced to succinate. The final reaction in the dicarboxylic acid pathway involves the decarboxylation of succinate, via three distinct steps, to propionate. This final reaction can occur in two ways typified by the systems of V. alcalescens (Delwiche, Phares and Carson 1956) in which the CO_2 from the decarboxylation step is readily released, and of propionibacteria wherein a biotin-containing transcarboxylase transfers the "C_1" derived from methylmalonyl-CoA to pyruvate thus completing

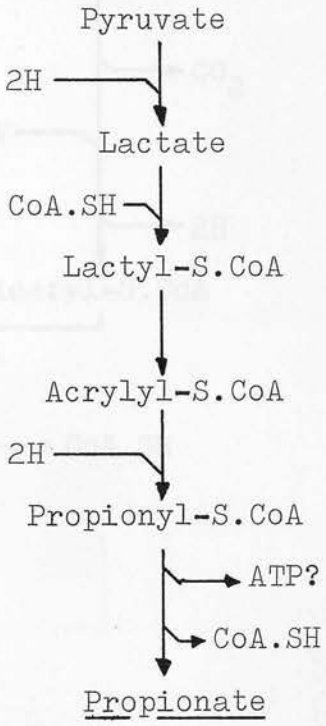
a cyclic pathway (Stjernholm and Wood 1963). The cyclic operation of the dicarboxylic pathway as represented in Fig. 4a does not require an energy source (ATP), whereas the non-cyclic pathway utilising a pyruvate carboxylation system (not the malic enzyme) in the initial reaction requires an energy source (Baldwin 1965). No energy-yielding steps are known for this pathway but the transfer of electrons from NADH to fumarate via fumaric reductase does involve a free-energy change sufficient to bring about phosphorylation (Singer, Kearney and Massey 1957).

The direct reductive pathway involves activation of lactate to lactyl-CoA, dehydration of lactyl-CoA to acrylyl-CoA and a reductive step resulting in propionyl CoA (Fig. 4b). The conversion of propionyl-CoA to propionate releases CoA which can, through a CoA transphorase, enter the initial step of the pathway to activate lactate without the use of ATP (Baldwin 1965). ATP formation during this pathway has not been shown but as with the dicarboxylic pathway, may be inferred, probably at the step of acrylyl-CoA reduction.

Butyrate, and higher fatty acids, have been shown to arise by two alternative pathways. One involves the reversal of β-oxidation (Fig. 5) and the other the formation of malonyl-CoA as an intermediate (Fig. 6). There is evidence to suggest that butyrate is synthesised via the reversal of β-oxidation and higher fatty acids by

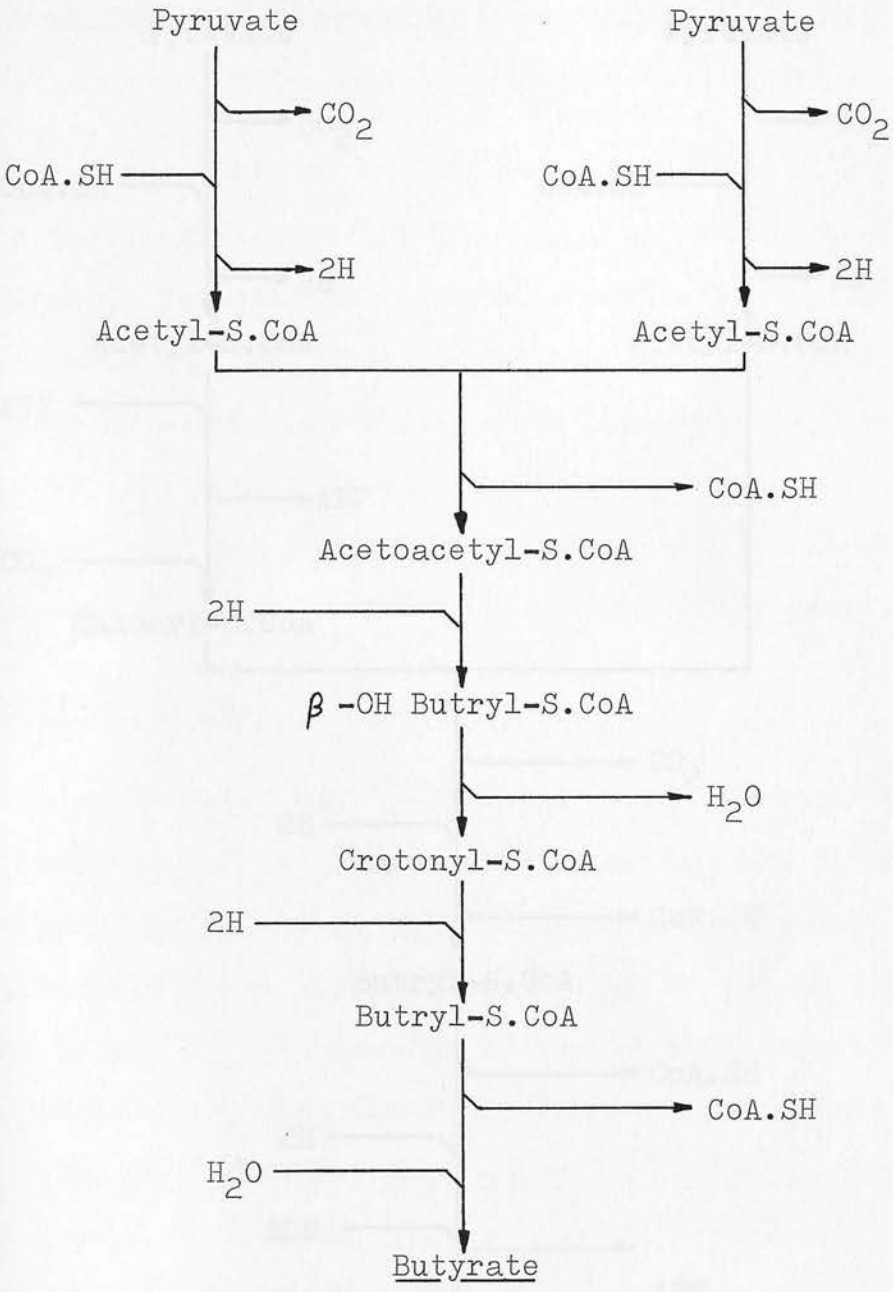


a) Dicarboxylic acid pathway.



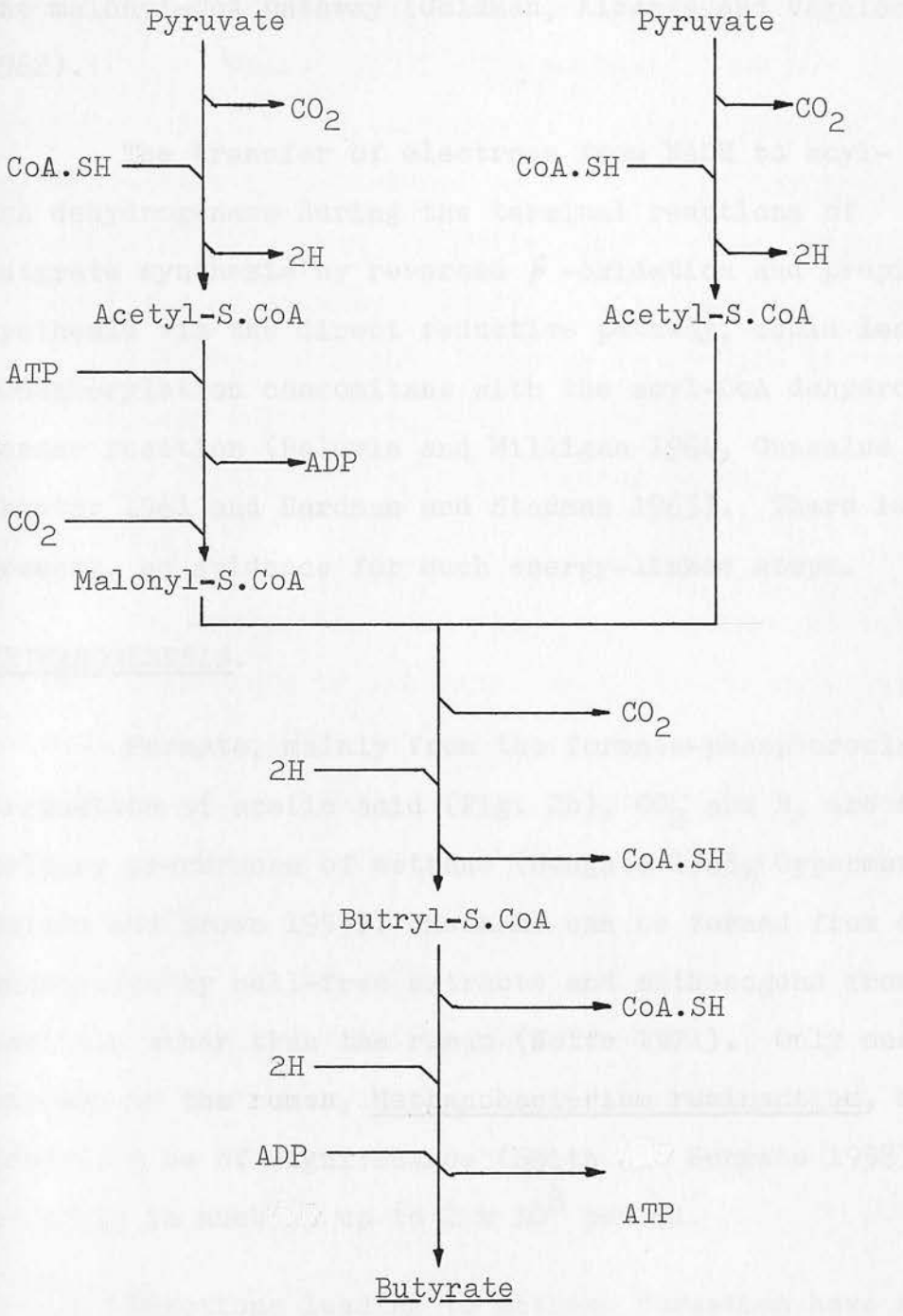
b) Direct reductive pathway.

Fig. 4 Pathways of propionate formation.



[Overall : 2 Pyruvate ⟶ Butyrate + 2 CO_2]

Fig. 5 Pathway of butyrate formation by reversal of β -oxidation.



[Overall : 2 Pyruvate ⟶ Butyrate + 2 CO_2]

Fig. 6 Pathway of butyrate formation via malonyl-CoA.

the malonyl-CoA pathway (Goldman, Alberts and Vagelos 1962).

The transfer of electrons from NADH to acyl-CoA dehydrogenase during the terminal reactions of butyrate synthesis by reversed β-oxidation and propionate synthesis via the direct reductive pathway, could lead to phosphorylation concomitant with the acyl-CoA dehydrogenase reaction (Baldwin and Milligan 1964, Gunsalus and Shuster 1961 and Hardman and Stadman 1963). There is, at present, no evidence for such energy-linked steps.

METHANOGENESIS.

Formate, mainly from the formate-phosphoroclastic production of acetic acid (Fig. 2b), CO_2 and H_2 are the primary precursors of methane (Hungate 1963, Oppermann, Nelson and Brown 1959). Methane can be formed from other substrates by cell-free extracts and methanogens from habitats other than the rumen (Wolfe 1971). Only one species in the rumen, Methanobacterium ruminantium, has proved to be of significance (Smith and Hungate 1958) occuring in numbers up to 2×10^8 per ml.

Reactions leading to methane formation have not been resolved, although the terminal reaction involving demethylation of methylcobalamin (methyl-Co-5,6-dimethylbenzimidazolcobamide; CH_3-B_{12}) has been demonstrated with cell-free extracts of Methanobacterium (Wolfe 1971). These reactions require ATP, in catalytic

rather than substrate amounts, for methane formation (Roberton and Wolfe 1969). The mechanism for ATP generation in methane bacteria is unknown though a linear relationship between methane and ATP production has been demonstrated (Roberton and Wolfe 1970). There is also evidence for an enzymic cofactor, designated coenzyme M (Co-M), involved in methyl transfer in methane bacteria. Co-M has been detected only in methane bacteria and has been shown to serve as a growth factor (McBride and Wolfe 1970, 1971).

The production of methane by cell-free extracts and in rumen fluid is inhibited by methylene chloride, chloroform and carbon tetrachloride (Bauchop 1967, Wood, Kennedy and Wolfe 1968). These compounds react chemically with B_{12} vitamins to form chloromethylcobalamins; kinetic studies indicate that the inhibition is competitive (Wood et al. 1968). It has been calculated that approximately 8-10% of energy consumed by ruminant animals is lost as eructed methane (Blaxter 1962) and compounds effective in inhibiting methanogenesis have received attention in recent research. This topic is dealt with in a later section (D).

NITROGEN METABOLISM.

PROTEIN In general, proteins entering the rumen are broken down to peptides and amino acids. The main proteolytic species are among the genera Bacteroides, Selenomonas and Butyrivibrio (Blackburn 1965) and protozoans such as Entodinium (Blackburn and Hobson 1960).

Blackburn and Hobson (1962) found that proteolytic acitivity was restricted to 10% of their rumen isolates. The digestibility of proteins has been related to their solubility (Henderickx and Martin 1963); a relationship for which Blackburn (1965) found little evidence with pure cultures.

AMINO ACIDS. Amino acids from breakdown of dietary protein, together with those introduced directly in the diet, are largely metabolised by the rumen microflora with a consequent production of carbon dioxide, ammonia and low-molecular-weight acids. The proportions of these products depends upon the particular amino acids being degraded (Lewis 1955) and the enzymic mechanisms of the breakdown. The deamination of aspartate, for example, results in fumarate, while oxidative deamination of glutamate yields α-keto glutarate. These compounds are not terminal products; they have not been identified in the rumen in significant quantities and almost certainly become incorporated in other pathways such as those leading to propionate. Several low-molecular-weight acids have been identified, for example iso-butyric, iso-valeric and 2-methyl butyric acid (El Shazley 1952, Annison 1954, Woods and Luther 1962, Bath and Rook 1965). Oxidative deamination of valine, leucine and iso-leucine respectively would result in these compounds.

Barber (1961) demonstrated, that clostridial fermentation of labelled α-amino valeric acid resulted in

either valeric acid by reductive deamination, or propionic and acetic acids by β-oxidation. Incubation of L-lysine with rumen fluid produced butyric acid, acetic acid and ammonia.

Deamination by pure cultures of rumen micro-organisms has received little study. Ruminal protozoa have been shown to deaminate amino acids (Warner 1956). Lewis and Elsden (1955) demonstrated the deamination of L-cysteine, L-serine and L-threonine by Selenomonas ruminantium and Peptostreptococcus elsdenii.

NON-PROTEIN NITROGEN Ruminal ammonia can arise from non-protein nitrogen as a result of reduction or hydrolysis. Nitrate introduced into the rumen is reduced to ammonia (Lewis 1951 [a,b]). Vibrio succinogenes has been shown to carry out this reduction (Wolin, Wolin and Jacobs 1961).

Urea is hydrolysed to ammonia and CO_2 in the rumen (Pearson and Smith 1943) by intracellular bacterial ureases (Jones, MacLeod and Blackwood 1964). Jones et al. found that about 0.3 of their isolates produced urease. There is no evidence of urease production by ruminal protozoa (Abou Akkada and Howard 1962).

Other compounds from which ammonia has been shown to be produced are guanine, hypoxanthine, xanthine, uric acid, uracil and thymine (Jurtshuk and Hueter 1955).

MICROBIAL PROTEIN SYNTHESIS Pearson and Smith (1943)

and Smith and Baker (1944) showed microbial protein synthesis *in vitro* with a concomitant fall in non protein nitrogen. The ratio of organic matter fermented in the rumen, to protein synthesized is about 10:1 (Hungate 1966). Rapid incorporation of ^{15}N labelled substrates by bacteria and protozoa, appearing at maximum concentrations after 5 and 6 days respectively, was shown by Ulbrich and Scholz (1966).

The nature of carbohydrate fermented influences the utilisation of nitrogen for bacterial protein. Starch and other polysaccharides result in reduced ammonia concentrations (Warner 1956) due to increased utilisation of ammonia for protein synthesis. Similarly, Ely, Little, Woolfolk and Mitchell (1967) found that, on a high ratio of starch to cellulose, 30% of dietary zein was converted to microbial protein whereas 26% was incorporated when the ratio was reversed. Abou Akkada and Balckburn (1963) found ammonia to be used in preference to amino acids by most of their proteolytic isolates. Bryant and Robinson (1962), working with pure cultures found that half of their strains utilised either ammonia or amino acids, and a quarter of them required only ammonia. An absolute requirement for amino acids was shown by 6% of their isolates.

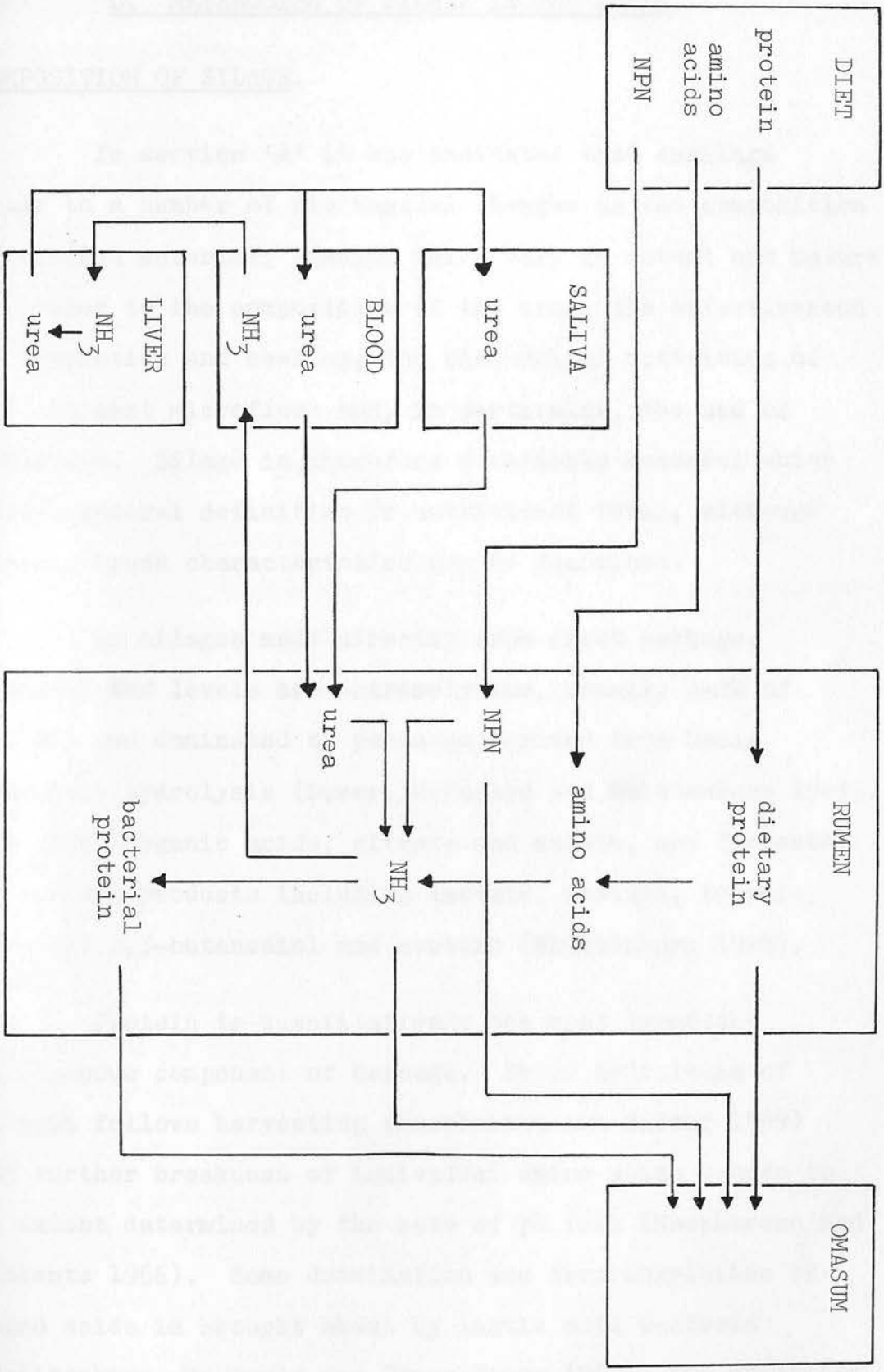


Fig. 7 Fate of dietary nitrogenous compounds in the ruminant.

C. METABOLISM OF SILAGE IN THE RUMEN.

COMPOSITION OF SILAGE.

In section 'A' it was indicated that ensilage leads to a number of biochemical changes in the composition of ensiled material; changes which vary in extent and nature according to the composition of the crop, the effectiveness of compaction and sealing, the biochemical activities of the dominant microflora and, in particular, the use of additives. Silage is therefore a variable material which defies general definition in nutritional terms, although certain broad characteristics may be described.

In silages made directly from fresh herbage, residual WSC levels are extremely low, usually 1-2% of the DM, and dominated by pentoses derived from hemicellulose hydrolysis (Dewer, McDonald and Whittenbury 1963). The plant organic acids, citrate and malate, are fermented to various products including lactate, acetate, formate, ethanol, 2,3-butanediol and acetoin (Whittenbury 1968).

Protein is quantitatively the most important nitrogenous component of herbage. Rapid hydrolysis of protein follows harvesting (Macpherson and Slater 1959) and further breakdown of individual amino acids occurs to an extent determined by the rate of pH fall (Macpherson and Violante 1966). Some deamination and decarboxylation of amino acids is brought about by lactic acid bacteria (Whittenbury, McDonald and Bryan-Jones 1967), but extensive

degradation only occurs when an adequately low pH value is not achieved (Gibson 1965).

The use of inhibitory additives can have a marked effect on the composition of silage. In silages made with the addition of 1.7% (w/w forage DM) formaldehyde, Barry and Fennessy (1972) found NH_3-N (g/kg total N) and lactic acid (g/kg DM) of 145 and 23.2 compared with 65 and 8.3 in untreated silages.

NUTRITIONAL VALUE OF SILAGE.

The nutritional value of foodstuffs depends mainly on the extent of digestion (digestibility), the efficiency of utilisation of digested components and the quantity ingested (McDonald, Edwards and Greenhalgh 1973).

Digestibility of silage organic matter has been shown to be comparable with that of the original grass (Harris and Raymond 1963)

Utilisation of digested nutrients is influenced by the type of fermentation prevailing in the rumen. Ruminal end products of digestion in which the proportion of acetate is high are used less efficiently than those with a high proportion of propionate (Blaxter 1967). Since diets rich in soluble carbohydrate tend to result in high proportions of propionate (McDonald et al. 1973), the low residual WSC of fermented silage could give rise to an acetate biased fermentation with consequent poor utilisation of products. Anderson and Jackson (1971) obtained higher proportions of

ruminal acetate on silage than fresh grass, but the differences were not great and further evidence, supporting this observation, is desirable.

Intake is influenced, in monogastric animals, by chemostatic and physiological mechanisms which Jones (1972) considered applicable to ruminants on high digestibility diets. With fibrous foods of lower digestibility, intake is determined mainly by rate of digestion, and hence rate of passage, in the rumen. Crampton (1957) suggested that voluntary intake of forages is limited by rate of cellulose and hemicellulose digestion, factors influenced by lignification of mature forages or partial deficiency of nitrogen for the rumen organisms. It is known, however, that this does not apply to silages since they normally have DM intakes well below those obtained for fresh or dried herbages of comparable digestibility.

Increased DM content of silages was shown by Thomas, Moore, Okamoto and Sykes (1961) and Jackson and Forbes (1970) to result in a linearly related increase in DM consumption by ruminants. This phenomenon has not been satisfactorily explained.

Various appetite-depressants such as aldehydes, histamines and organic acids may be present in larger concentrations in wet silages (Neumark, Bondi and Volcani 1964; Harris, Raymond and Wilson 1966). Other possible causes of limited silage intake are products of clostridial

protein-degradation (observed as volatile N) or high concentrations of free acids in silages of low pH (McLeod, Wilkins and Raymond 1970; Wilkins, Hutchinson, Wilson and Harris 1971; Wilkins and Wilson 1971). Increased DM intake of low pH (4.1) silage was obtained by McLeod et al. (1970) after increasing the pH to 5.4 by the addition of sodium bicarbonate. Addition of lactic acid was found to decrease voluntary intake. Orth and Kaufman (1966) observed a depression in salivary secretion when acids were infused into the rumen; suggesting that salivary-flow may be a factor related to intake, perhaps by an effect on ruminal pH.

High soluble-N content could result in limited nitrogen availability to rumen organisms and this, together with high ruminal ammonia concentrations measured on silage diets (Chalmers 1963), is consistant with the earlier noted conclusions of Crampton (1957).

EFFECT OF SILAGE ON THE RUMEN FERMENTATION.

pH. Christian and Williams (1957) reported ruminal pH values of 7.15 and 6.7 before feeding, and minimal values of 6.7 and 6.6 with sheep on diets of fresh and dried grass respectively. These workers later reported values of 7.2 - 7.6 at 2 hours post-feeding in a similar experiment with a range of silage diets (Williams and Christian 1959). Anderson and Jackson (1971) measured ruminal pH in sheep offered grass, unwilted and wilted silages and hay. Values ranged from 6.02 to 7.02 but no association between

particular values and specific diets was shown.

VFA. Williams and Christian (1959) reported total VFA (TVFA) values, in the rumen of sheep at 2 hours post-feeding, from 49 to 87m mol/l for 12 silages of varying organic matter digestibilities. There was no correlation between organic matter digestibility and TVFA.

Relationships between molar proportions of VFA and feed constituents have been found: Bath and Rook (1961) and Terry and Tilley (1961) established an inverse relationship between WSC and molar proportion of acetic acid.[1] A correlation has also been shown to exist between the proportion of acetic acid and the fibre content of some diets (Elliot and Loosli 1959; Bath and Rook 1963).

There is little information on the effects of silages on ruminal concetrations of VFA. Balch and Rowland (1957) reported ruminal molar percentages of 73.7, 16.8 and 6.6 for acetic, propionic and butyric acids with a silage diet and Tilley, Derias and Terry (1960) reported values of 61, 20 and 19.[2] Hay, silages and dried grasses were all found by Bath and Rook (1965) to give characteristically high proportions of acetic acid whilst the silages only, gave lower proportions of butyric acid together with higher proportions of branched-chain acids. Mahapatro and Leffel (1964) found lower acetate and higher propionate and butyrate levels with hay and wilted silages, compared with wet silages.

---

1. Measured with a grass diet
2. Percentages based on concentration, not molarity

Anderson and Jackson (1971) found a tendancy for unwilted silages to result in higher levels of ruminal acetate and lower propionate than grass, wilted silage or hay. They also found a significant direct relationship between dietary crude fibre and ruminal acetate and an inverse relationship between fibre and propionate.

AMMONIA. Ruminal ammonia concentrations can vary widely; Briggs, Hogan and Reid (1957) recorded values of 2.0 to 223 mg NH_3-N/100 ml using sheep on a range of diets including roughage alone, roughages supplemented with carbohydrate concentrate and roughages supplemented with carbohydrate and protein concentrate. Williams and Christian (1956) reported a range from 9.1 to 46.6 mg/100 ml for grazing sheep, while ten silage diets gave values from 12.9 to 32.5 mg/100 ml (Williams and Christian 1959).

There is evidence that levels of ammonia in the rumen are characteristically high with silage diets (Fatianoff, Durand, Tisserand and Zelter 1966; Durand, Zelter and Tisserand 1968).

MICROBIOLOGY. Whilst present knowledge of rumen microbiology is limited, the sum of information in the case of silage diets is negligible. Direct comparisons with other diets have not been made, and the only information available is that published by Williams and Christian (1959) who made direct microscopic counts. The range of total numbers observed (circa 10^{10}/ml) and

gross morphological types described did not indicate any major influence of silage composition. The authors did acknowledge that large species differences, undetectable by the methods used, may have been induced. Average counts for sheep, offered dried grass from the same pasture were found to be higher.

D. EFFECTS OF SPECIFIC COMPOUNDS ON THE RUMEN AND RUMEN MICROFLORA.

COMPOUNDS ACTIVE IN REDUCING METHANOGENESIS.

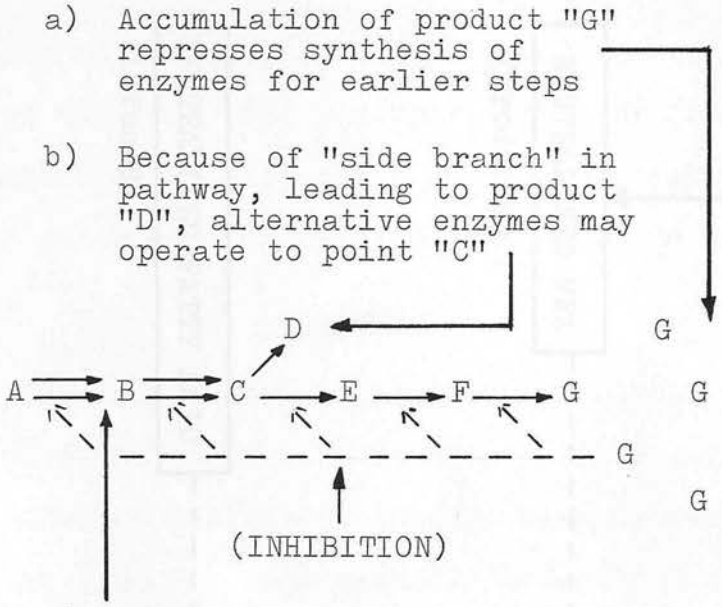
Much research has been applied to areas of rumen metabolism related to the production of methane and justified by the overall loss of energy resulting from this production (Czerkawski 1973). The addition, either by direct infusion or by incorporation in the diet, of metabolically active compounds which restrict methanogenesis, has proved effective. Two types of such compounds have been considered, those offering alternative hydrogen acceptors to replace CO_2 and produce a non-gaseous product, and those active by inhibition.

The addition of unsaturated fatty acids to the rumen has been shown to depress methane production to an extent greater than could be accounted for by saturation of all available double bonds (Czerkawski, Blaxter and Wainman 1966 [a,b]). These experiments showed that even saturated long chain fatty acids and their sulphated derivatives depressed methane production, and, to some extent, cellulose digestion. It was concluded that the effect was probably due to the surface-active properties of these compounds.

Reduction in methane production appears to be associated with an increase in the proportion of propionic acid in the fermentation products (Demeyer, Van Nevel, Henderickx and Martin 1969; Shaw and Ensor 1959), an

observation consistant with the theoretical fermentation balance since pathways leading to propionic acid are net "consumers" of hydrogen.

Studies with pure cultures of rumen organisms appear to support the above findings (Henderson 1973), since organisms which produce propionic acid are less affected by fatty acids. Some organisms show a dualistic response, for example Butrivibrio B835 is stimulated by low concentrations of fatty acids, but inhibited at higher concentrations. The effect of a supply of fatty acids, in reducing the need for de novo synthesis, was suggested by Henderson (1973) as an explanation for stimulation at low concentrations. Inhibition at higher concentrations was thought to be due to surface coating of the bacterial cells by long-chain surfactant molecules, an explanation also offered by Czerkawski, Blaxter and Wainman (1966[c]). No theory has been offered, however, to account for the avoidance of this phenomenon by the propionic acid producing organisms. An alternative explanation of the effect of fatty acids may be "end product inhibition" (White, Handler and Smith 1968) as illustrated in Fig. 8. This concept involves reactions being rate controlled by the concentrations of their products in the medium; the ruminal production of VFA, for example, is limited by their accumulation (Stranks 1956). Compounds structurally analagous with an end product will, in some cases, also bring about a degree of inhibition, a fact which has found application in the direct inhibition of methane production by halogenated methane analogues



(Based on White, Handler and Smith 1968)

Fig. 8 End product inhibition.

(Clapperton and Czerkawski 1972, Prins, Van Nevel and Demeyer 1972). The fatty acids used in the work already discussed are analogues of ruminal VFA and their presence in the rumen could cause a measure of inhibition as indicated in Fig. 9. Production rates of VFA were not measured in the experiments of Czerkawski, Blaxter and Wainman (1966 [a,b,c]) and control diets, without additives, were not offered by Shaw and Ensor (1959). It is not clear therefore, if the reduction in methane production was related to a fall in VFA production although Czerkawski, Blaxter and Wainman (1966 [c]) did observe a depression of cellulose hydrolysis when methane production was inhibited. Whilst the attenuation of cellulose hydrolysis was about half that observed in the

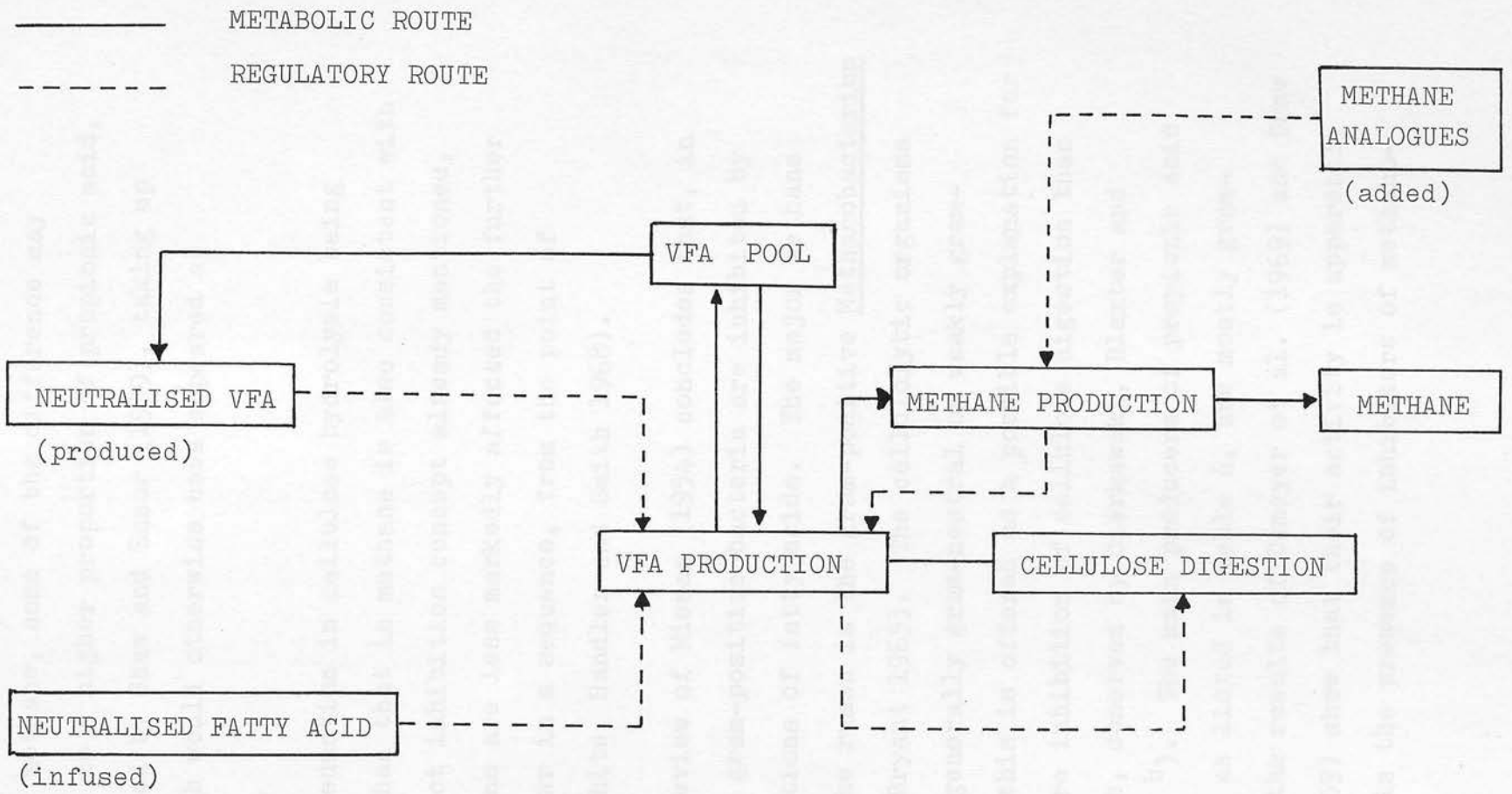


Fig. 9 Suggested mechanism of inhibition by compounds added to the rumen.

production of methane, some of the difference may have been due to a higher proportion of propionic acid, as demonstrated by Shaw and Ensor (1959), taking up hydrogen which would otherwise have appeared as methane.

The reduction in cellulose hydrolysis being less marked than that in methane is also consistent with the end product inhibition concept already mentioned, since reactions are less markedly affected the further back they occur in a sequence, from the point of inhibition (White, Handler and Smith 1968).

The review of Nieman (1954) concludes that, in general, only gram-positive bacteria are inhibited by low concentrations of fatty acids. The major methane producer in the rumen is the gram-positive Methanobacterium ruminantium (Bryant 1965). The cellulolytic organisms are however, generally gram-neutral or weakly gram-positive and this is offered as a possible explanation for the less severe inhibition of cellulose digestion than methanogenesis, observed by Czerkawski, Blaxter and Wainman (1966 ^{b}). The main producers of propionic acid in the rumen, as listed in Table 6, are mostly gram-negative and the results of Demeyer et al. (1969) and Shaw and Ensor (1959) show that their activity is apparently undiminished in the presence of inhibitors of methano-

Table 6 Propionate Producing Organisms.

	Propionate Production	Morphology	Substrate	Gram
Succinomonas amylolytica	+	coccoid	starch	-
Veillonella alcalescens	+	coccal	lactate	-
Anaerovibro lipolytica	++	rod	lipids	-
Peptostreptococcus elsdenii	+	coccal	lactate	+
Selenomonas ruminantum	++	crescent	most	-
Selenomonas lactilytica	+	crescent	lactate	-

Based on Hungate (1966), Bryant (1959) and "Bergy" (1973).

genesis. These factors considered together tend to point to the conclusion that the effects of fatty acids in the rumen are due to selective toxicity although this may take the form of differing degrees of surfactant effects on the cell walls of gram-positive and gram-negative bacteria. One of the most prolific producers of propionic acid is the lipolytic organism Anaerovibrio lipolytica (Hobson and Mann 1961) which might make a significant contribution to increased propionate when fatty acids are added to the diet.

The increased proportion of propionate can also be explained in terms of end product inhibition since propionic acid producers, which do not usually employ cellulose as a primary substrate (Table 6), will be less affected than other organisms by conditions where cellulose digestion is diminished by the accumulation of metabolic hydrogen not removed as methane. Cellulolytic organisms tend to be producers of acetate (Henderson 1973) and their inhibition would also increase the proportion of propionate.

The explanation of the inhibitory effects of fatty acids being a consequence of their surface-active properties (Henderson 1973; Czerkawski, Blaxter and Wainman 1971 [c]) requires further elucidation since the salts of fatty acids are anionic detergents which tend to be repelled by the net negative charge of bacterial cells (Davis, Dulbecco, Eisen, Ginsberg and Wood 1968). It is known that such

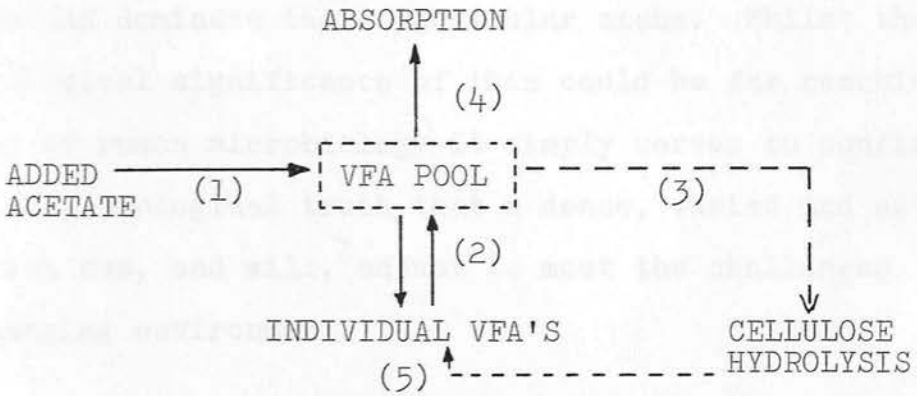
compounds, in solution, are moderately bactericidal to gram-positive organisms and less so to gram-negative, although where there is an insoluble lipid phase, surface coating of the cells may arise. This would be detrimental to the function of the cell (though less so to lipolytic types than others) but important only at high concentrations and unlikely to account for the results obtained in the work cited here.

Other compounds have been incorporated into ruminant diets, to suppress methane production. It has been shown (Trei, Parish, Singh and Scott 1971; Trei, Singh and Scott 1970) that either trichloroacetamide or a hemiacetal compound of starch and chloral hydrate will suppress methane production, reduce the ratio of acetic acid to propionic acid and increase the energy retained by the animals. The similar effect of chloroform has already been mentioned (Clapperton and Czerkawski 1972) and it is apparent that these compounds, incorporating carbon atoms with trichloro substitution, are effective suppressors of methane production, probably by inhibition as end-product analogues.

COMPOUNDS HAVING OTHER EFFECTS.

The changes induced in the rumen by the addition of the lower fatty acids (C_2, C_3 and C_4) have also been studied (Griffiths 1971). Addition of a single acid generally causes an immediate increase in its concentration followed by a decline to the pre-addition concentration after

about four hours. The concentrations of VFA in the rumen appear to be self stabilising, this being brought about by the considerable interconversion which occurs (Leng 1969). The size of the VFA "pool" will be determined by production rate, which is itself a function of substrate concentration, and absorption rate. The end-product inhibition effects already discussed are likely to play a part in this and the scheme in Fig. 10 is proposed to describe the situation.



(1) Added acetate contributes to VFA pool.

(2) Pool size increases, interconversions restore proportions.

(3) Cellulose hydrolysis is reduced by increased VFA concentration.

(4) VFA absorbed reduces size of pool.

(5) Individual VFA's again produced by cellulolysis as pool size declines.

Fig. 10. Depression of cellulolysis and VFA production by end product inhibition.

Certain additional groups of compounds have also been studied, notably antibiotics and herbicides. Antibiotics have been added to ruminant diets to increase

weight gains and have also been suggested as a treatment for bloat (Shellenberger, Jacobson, Hartman and McGillard, 1964). The effects of antibiotics on rumen function have been investigated by El Akkad and Hobson (1966). Rumen bacteria with no previous history of contact with antibiotics show susceptibility to a wide range of these compounds (Fulghum, Baldwin and Williams 1968). Pure cultures were shown to contain some antibiotic-resistant cells, however, and in vivo such cells could presumably flourish and dominate their particular niche. Whilst the pharmacological significance of this could be far reaching, in terms of rumen microbiology it simply serves to confirm the general ecological truth that a dense, varied and active population can, and will, adjust to meet the challenges of a changing environment.

The fate, in the rumen, of certain herbicides has been investigated to establish whether they are degraded by the rumen microflora and for possible effects, on the microflora. Trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine), Mobam (Benzo(b)thein-4-yl methylcarbamate) and Propazine (2-chloro-4,6-bis (isopropylamino)-s-triazine) have been studied (Williams and Feil 1971; Williams and Stolzenberg 1972; and Williams, Davison and Thacker 1968). In general, results of these experiments showed that these compounds were not degraded by the rumen microflora and did not cause inhibition.

The urease inhibitor, acetohydroxamic acid, has been shown to affect the growth and VFA production of

rumen bacteria. Both pure culture studies (Chan and Jones 1973) and mixed culture studies (Jones 1968) revealed that there were effects, other than urease inhibition, which could be ascribed to acetohydroxamic acid. The use of this compound to provide a slow, controlled release of urea and ammonia from a urea supplemented diet, could therefore be accompanied by a long-term disturbance of other aspects of ruminal function.

E. TECHNIQUES IN RUMEN MICROBIOLOGY

CULTIVATION OF RUMEN ORGANISMS.

The microflora of the rumen was among the last of the major groups of micro-organisms to be grown in artificial culture. According to Hungate (1950) most of the difficulties encountered by early workers appear to have arisen from failure to achieve complete anaerobiosis even though the technology of anerobic culture was well established. Since the most singular property of the rumen microflora considered en masse is its ability to digest cellulose, early interest concentrated on cellulolytic organisms. According to Hungate (1966), attempts to cultivate these organisms were frustrated for the following reasons : 1) absence from the medium of certain growth factors (CO_2, n-valeric acid and C_4 and C_5 branched acids), 2) failure to achieve sufficient anaerobiosis, 3) excessive concentrations of cellulose, 4) unsuitable cellulose preparations.

The first modern attempts at culturing rumen micro-organisms were those of Gall, Stark and Loosli (1947) who employed media, containing peptones and other organic extracts, for culture counts. Hungate (1947, 1950) introduced a medium containing rumen fluid and a bicarbonate/CO_2 buffer together with reducing agents. Kistner (1960), Bryant and Robinson (1961), Bryant (1963) and Caldwell and Bryant (1966) and later workers have subsequently used this medium in conjunction with techniques ensuring the exclusion of oxygen. Typical

modern formulae of such media are presented by Latham and Sharpe (1971) and Hobson and Mann (1970).

MEDIA FOR RUMEN BACTERIA.

Hungate (1966) distinguished two types of medium for the cultivation of bacteria: 1) habitat-simulating media which attempts to support all the types of organism present in a particular habitat, 2) niche-simulating media which are employed for the isolation of restricted groups of bacteria.

Habitat-simulating media for rumen organisms generally contain filter-sterilized rumen fluid or, in the case of more recently developed formulae, the essential accessory nutrients as part of a "defined" medium (Caldwell and Bryant 1966). Media without rumen fluid but containing hemin, trypticase, yeast extract and a VFA mixture, have given colony counts similar to those obtained with media based rumen-fluid (Caldwell and Bryant 1966). Media of both kinds also contain inorganic salts, the concentrations in most cases being similar to those used in the original medium of Hungate (1947).

Niche-simulating media are employed for the isolation and/or enumeration of particular groups of bacteria. The degree of selectivity which can be achieved varies according to the specific niche being "simulated" and highly selective media cannot be devised in all cases

(Hungate 1962). The use of rumen fluid in such media tends to reduce its selectivity because it contains, even in limited concentrations, all nutrients required by rumen organisms (Hungate 1966). The selective properties of such media are generally based on the nature of the energy-containing substrate; cellulose (Hungate 1947), starch (Hamlin and Hungate 1956), casein (Blackburn and Hobson 1960) etc.

ANAEROBIOSIS

Media for rumen bacteria and protozoa invariably contain reducing agents, a mixture of cysteine HCl and sodium sulphide (0.015% w/v, each), being used routinely for most media (Latham and Sharpe 1971). Cysteine HCl (0.05% w/v) is used in media for the detection of sulphide production. Reduction indicators are also incorporated into media, resazurin (0.0001% w/v) being quoted by most authors although it is not an ideal indicator of reduction since the redox couple of the reaction of resorufin (the initial reduction product of resazurin) to dihydroresorufin has the relatively high Eo' of -0.042 volts at pH 6.87 (Twigg 1945). Other indicators with redox couples at lower Eo' values are reported to be toxic unless used at dilutions which prevent the observation of colour changes (Bryant 1963).

Methods used in the preparation, dispensing, inoculation and incubation of media for rumen organisms are intended to achieve and maintain reduced conditions

since many rumen bacteria are killed by oxygen (Giescke 1960) and are unable to reduce a medium themselves (Hobson and Mann 1970). Techniques capable of fulfilling these requirements have been developed from the pioneering work of Hungate (1947, 1950) and two distinct approaches may now be recognised; the "open-tube" technique and the "closed tube" technique. In the closed-tube method, the bung is never removed and inoculation is performed via injection with a hypodermic syringe, whereas with the open-tube method all inoculation and transfers, although still performed anaerobically, take place with the bung removed. The closed-tube technique has less inherent danger of oxidation or contamination but for routine laboratory use, especially when dealing with large numbers of cultures, the open-tube method is considered to be more practicable (Latham and Sharpe 1971).

Similarly, various sophisticated methods for dispensing highly reduced media have been described, for example, Williams (1966). Although dispensing is the most difficult and tedious part of the whole anaerobic procedure, many workers claim that "hand" operations based on the open-tube technique are still the only way to maintain reduced conditions (Hobson and Mann 1971; Latham and Sharpe 1971).

The basis of the open-tube technique is the constant application of a stream of O_2-free gas *via* a fine bore stainless steel tube to the contents of all media vessels whilst the bung is removed. Detailed descriptions of these techniques are given by Hungate (1966), Hobson and Mann

(1970) and Latham and Sharpe (1971).

ENUMERATION OF RUMEN MICROBES

Rumen organisms have been counted by both culture counts, in which only viable organisms capable of growth on the medium employed may be counted, and direct counts, in which all microscopically recognisable organisms, living and dead, are included. Both habitat and niche-simulating media may be used in making cultural counts and in some work both types of media have been employed, to provide a cross-reference (Hungate 1962). Culture counts of organisms from specific niches have been made directly in niche-simulating media (Hungate 1947, Hamlin and Hungate 1956, Blackburn and Hobson 1960) and indirectly by subculturing and testing for specific properties after initial isolation on non selective (habitat-simulating) media (Bryant, Small, Bouma and Robinson 1958). Van Gylswyk (1970) compared these approaches in the case of cellulolytic rumen bacteria and found that similar results were obtained in each case. A major problem associated with differential counts of organisms from various niches detected by biochemical reactions in specific media, is interpretation of the results, which can lead to discrepancies in counts between different observers (Hobson and Mann 1971).

A method for culture enumeration of rumen bacteria on a micro scale, using heat-sealed capillary tubes containing medium, has been described by Claypool, Jacobson and Wiseman (1961).

Direct counting of rumen bacteria and protozoa under the microscope, with suitable counting chambers, has been described by Warner (1956 and 1962[a]). Chambers with a depth of 0.2mm have been employed for protozoal counts whilst 0.02mm deep units have been used when bacteria were enumerated. As an alternative to counting chambers, Hungate (1957) used known amounts of diluted rumen content smeared over a known area of a microscope slide, then dried and stained. Conventional direct counting techniques only allow differentiation of organisms with distinctive morphology (Warner 1962[b]) but the fluorescent antibody technique has been used for direct counts of serologically specific types of rumen bacteria by Hobson and Mann (1955, 1957).

F. THE IN VITRO RUMEN.

GENERAL.

The use of in vitro techniques in ruminant research is well established. For almost 40 years a variety of in vitro methods have been used (Woodman and Evans, 1938) although some experiments were carried out much earlier (Hofmeister 1881). Certain techniques, particularly those of Tilley and Terry (1963) and Alexander (1969), are routinely applied in the nutritional evaluation of feedstuffs. It is convenient to divide in vitro techniques into two types, batch culture and continuous culture, the essential difference being that whereas the batch culture is provided only with an initial supply of substrate which is gradually depleted whilst waste products accumulate, the continuous system constantly replenishes the substrate and removes waste products. The latter technique does, of course, more closely simulate the natural rumen.

The theory of continuous culture, in the microbiological sense, was formalised by Monod (1950) and Novick and Szilard (1950) and is modelled around a culture system which fulfils the following criteria :

1. The culture is pure and subject to "perfect mixing".
2. The rate of inflow of medium is constant and matched by the outflow of effluent (ie the culture volume remains constant).

A full account of the mathematical treatment of the theory is given in Appendix 1.A Two important parametric conclusion arise as a consequence of the theory; 1) the rate of microbial growth in a continuous culture must equal the dilution rate of the culture and 2) the culture density is set by the concentration of the growth-limiting component of the substrate. An apparatus which allows these criteria to be met is a "chemostat"; Herbert, Phipps and Tempest (1965) have described the design and instrumentation of the chemostat.

The natural rumen does not fit the idealised continuous culture model, because its flow kinetics are non-linear. The divergence of the rumen from the theoretical continuous culture model has been discussed by Hungate (1966) who concluded that the value of the continuous "model" of the rumen lies not so much in direct applicability as in providing a basis for reference. In vitro systems of the continuous type may thus be further sub-divided into those which are truly continuous (that is, attempt to fulfil the theory of continuous culture) and those in which the substrate is added periodically (as in the natural rumen). Some continuous in vitro rumens have used a flow of artificial saliva into a culture of constant volume (Rufener, Nelson and Wolin 1963; Slyter, Nelson and Wolin 1964; Aafjes and Nijhof 1967), with periodic addition of substrate. Such an arrangement cannot give rise to steady-state kinetics.

IN VITRO RUMEN SYSTEMS.

In vitro systems descibed during the last 20 years are listed in Table 7. This list is not exhaustive, Louw, Williams and Maynard (1949) and Dawson, Ward and Scott (1964) have also described in vitro systems, but these were identical to others described in Table 7 (Warner 1956 and Davey, Cheeseman and Briggs 1960). The apparatus of Warner (1956) was based on that of Louw et al. (1949) and consisted of a semipermeable sac supported in dialysing solution and containing 50 ml of rumen liquor. A constant stream of N_2/CO_2 (95/5) was passed through the culture and pH adjustment was carried out "when necessary" by the addition of buffer solution. These were batch systems in which only one aliquot of substrate was made available (Fig. 11/1).

The first continuous culture system (one which could be maintained "indefinitely" by replenishment of substrate) was that of Adler, Dye, Boggs and Williams (1958), who applied chemostat principles to an impermeable in vitro rumen. The vessel was supplied with a stream of nitrogen and stirred magnetically; pH control was designed to be inherent by the use of a suitable substrate/buffer mixture but although this was a continuous culture apparatus, culture periods did not exceed 10 hours. (Fig. 11/2)

Davey, Cheeseman and Briggs (1960) described an apparatus similar to those of Louw et al. (1949) and Warner (1956) but with the significant refinement of a continuously

Table 7 In vitro rumen systems.

No.	A Reference	B Culture Vessel	C Permeability System	D Gas Supply	E Agitation System	F pH System	G Heating System	H Culture System
1	Warner 1956	dialysis sac	single aliquots of buffer	constant flow	none	buffer added "as necessary"	water jacket	batch/ periodic feed
2	Alder, Dye, Boggs & Williams 1958	glass vessel	none	constant flow	magnetic stirring	substrate/ buffer mixture	external heater	continuous/ short term
3	Davey, Cheeseman & Briggs 1960	dialysis sac	constant flow of buffer	constant flow	none	measurement system, no control	water jacket	periodic feed
4	Stewart, Warner & Seeley 1961	glass vessel	none	none	overhead stirring	none	water jacket	continuous steady state
5	Harbers & Tillman 1962	glass vessel	none	constant flow	none	none	internal heater	continuous steady state
6	Gray, Weller, Pilgrim & Jones 1962	glass vessel	dialysis coil on agitator	head space flow	reciprocating agitator	measurement system - manual control	water jacket	batch with buffer flow
7	Rufener, Nelson & Wolin 1963	plastics vessel	ion exchange resin in dialysis sac	none	rocking agitator	see column 'C'	water jacket	periodic feed buffer flow continuous
8	Slyter, Nelson & Wolin 1964	glass vessel	ion exchange resin in dialysis sac	none	magnetic stirring	see column 'C'	water jacket	periodic feed buffer flow continuous
9	Aafjes & Nijhof 1967	glass vessel	none	constant flow	reciprocating agitator with substrate bags	measurement with control, artificial saliva	water jacket	periodic feed continuous
10	Czerkawski & Breckenridge 1969	glass vessel	none	recirulation	none	none	water jacket	batch short term

Fig. 11 Key to symbols used.

A Agitator

B Buffer

C Culture

D Dialysate

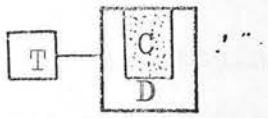
E Effluent

F Feed

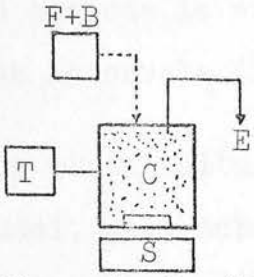
P Pump

S Stirrer

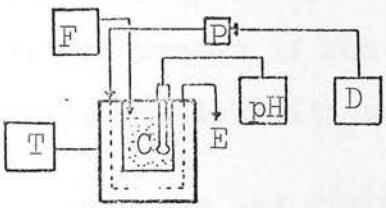
T Temperature control



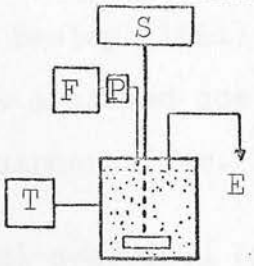
1 Warner (1956)



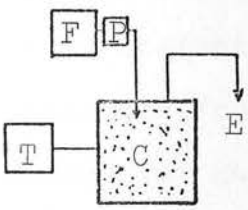
2 Adler et al. (1958)



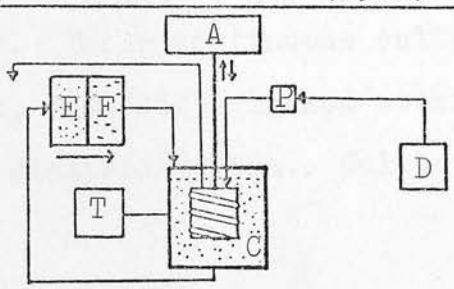
3 Davey et al. (1960)



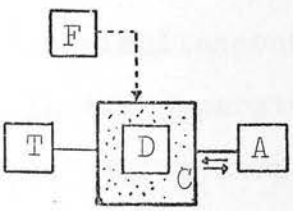
4 Stewart et al. (1961)



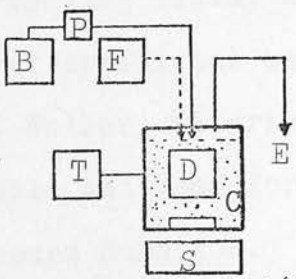
5 Harbers & Tilman (1962)



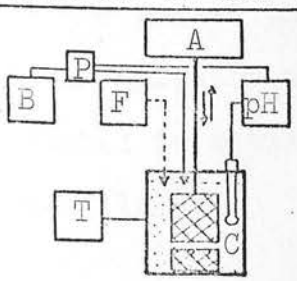
6 Gray et al. (1962)



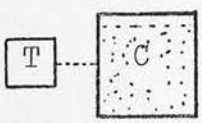
7 Rufener et al. (1963)



8 Slyter et al. (1964)



9 Aafjes & Nijhof (1961)



10 Czerkawski and Breckenridge (1969)

pumped dialysing solution which enabled high rates of waste-product diffusion to be maintained. This apparatus was truly continuous with culture periods in excess of 8 days. Feeding was periodic at 24h intervals (Fig. 11/3).

The attainment of a steady state culture (one which fulfils the continuous culture model, with substrate replenished constantly throughout the culture period) was attempted by Stewart, Warner and Seeley (1961) but culture periods in excess of 24h were not achieved due to poor mechanical reliability of the equipment (Fig. 11/4).

Harbers and Tillman (1962) described experiments with a batch culture system based on a permeable growth chamber and with a modified chemostat. Their continuous culture (modified chemostat apparatus, Fig. 11/5) lacked stirring, pH measurement/control and a dialysis system. Cultures were maintained for 24h only.

A double acting syringe which automatically maintained a constant culture volume by removing fluid, to compensate for the simultaneous addition of artificial saliva, was used in the apparatus of Gray, Weller, Pilgrim and Jones (1962). The system was permeable and used for batch-culture experiments of a few hours duration. Oxygen-free gas was passed through the culture-vessel headspace only and an agitator with vertical reciprocating action was attached to a wire mesh cage carrying the dialysis coils (Fig. 11/6).

The systems of Rufener, Nelson and Wolin (1963) and

Slyter, Nelson and Wolin (1964) were basically similar. Semipermeable sacs containing mixed ion-exchange resin were suspended in the culture fluid to absorb VFA and thereby control pH. Continuous flow of artificial saliva was maintained into the culture and "feeding" was periodic. Long term operation in excess of 200h was claimed for each of these machines which differed only in construction of their culture vessels and method of agitation (Figs. 11/7 and 11/8).

Aafjes and Nijhof (1967) devised a continuous-flow, periodic-feed system designed to simulate the effects of rumen motility. Two nylon bags were used, arranged one above the other in the culture vessel, with the upper bag attached to a reciprocating plunger, running at 5 strokes/m. During "runs" of 7 weeks the artificial rumen was "fed" twice a day. Every morning a bag filled with hay was attached to the plunger, whilst the bag of hay from the day before was placed at the bottom of the culture vessel. In the evening the remainder of the "ration" for that day was added to the contents of the bag attached to the plunger. Flow of rumen fluid through the plant material layer, as occurs in the natural rumen, was thus imitated. Saliva flow was provided automatically in response to pH drop and a constant volume maintained with a weir overflow (Fig. 11/9).

The apparatus of Czerkawski and Breckenridge (1969) was an impermeable, all glass, batch-culture system intended for experiments of short duration. Recirculation of the culture gas was brought about by a peristaltic pump in an

external loop arranged so that net gas production could be taken up in an adjustable expansion chamber maintained at constant pressure by reference to readings on an adjacent manometer.

ASSESSMENT OF ARTIFICIAL RUMENS

Warner (1956) suggested the following criteria for establishing the validity of "artificial rumens" :

1. Maintenance of numbers and normal appearance of the bacteria, selenomonads and protozoa of the rumen.
2. The maintenance of normal rates of digestion of cellulose, starch and protein, and of normal interactions between these.
3. The ability to predict quantitative results in vivo.

These criteria are based on comparison of in vivo and in vitro biochemistry and microbiology, taking no account of the extent to which the in vitro system simulates the natural rumen since, at least implicitly, accurate simulation is essential if they are to be fulfilled. Microbial populations are highly adapted to their environment and thus, rumen microbes can only, strictly speaking, be microbes in the natural rumen. According to this stringent ethnological definition, organisms from an "artificial rumen", regardless of their genealogy, are not actually rumen microbes. Conversely, if the environmental

simulation achieved by the in vitro apparatus is adequate, the microflora and microfauna it supports should be indistinguishable from that ot the natural rumen, even after several generations.

The artificical rumen of Warner (1956) was "biologically accurate" for periods up to 8 hours after inoculation. This is less than or equal to the theoretical mean generation time in the rumen (Hungate 1966). Over longer periods an increasing failure to meet the biological criteria given above was observed.

Amongst the other systems reviewed here, those where the culture period exceeded 10h (Table 7/3, 4, 7, 8 and 9) showed a diminution of protozoal numbers although the populations stabilised and were maintained at these lower levels. Several of the workers cited in Table 7 noted the poor accuracy with which the microflora and microfauna may be quantified and few have measured microbial numbers (Table 7/1, 3 and 7). Most of the systems cited were evaluated on the basis of biochemical parameters, particularly VFA concentrations and production rates. Table 8 lists the ranges of values observed for molar proportions of VFA in those of the cited systems for which results were presented. These data contain rather limited information since no account is taken of the different substrates used in each case. There are no clear patterns of results associated with permeable and impermeable systems and similar ranges of values arise with the batch and continuous

Table 8. Molar proportions of volatile fatty acids (VFA) in in vitro rumen systems.

No.	Reference	Molar Proportions (mol/100 mol VFA) of : acetic acid	propionic acid	butyric acid
3	Davey, Cheeseman and Briggs, 1960	46-54	19-27	20-24
4	Stewart, Warner and Seeley, 1961	58-63	20-24	11-17
6	Gray, Weller, Pilgrim and Jones, 1962	66-70	16-19	12-15
7	Rufener, Nelson and Wolin, 1963	64-70	17-21	13-15
8	Slyter, Nelson and Wolin, 1964	66-67	19-20	11-13
9	Aafjes and Nijhof, 1967	54-69	21-28	9-13
10	Czerkawski and Breckenridge, 1969	64-68	19-24	9-12

culture type. Gray et al. (1962) suggested, that as a stringent test for the artificial rumen, the molar proportions of the VFA should compare with those of the natural rumen on the same diet. Such comparative data are given by Davey et al. (1960), Gray et al. (1962) (permeable systems) and Aafjes and Nijhof (1967) (impermeable system) and are summarised in Table 9. These workers found good agreement between the composition of ruminal VFA fractions for in vivo and in vitro situations, although the impermeable system of Aafjes and Nijhof (1967) gave slightly lower values for acetate and higher for the remaining VFA's.

Data on VFA production rates are also sparse and, where they are presented, much relevant detail is often omitted. Table 10 gives data provided by authors, and data which can be derived from information presented in the respective papers. The figures in Table 10 for VFA production rates in the cases of Warner (1956) and Harbers and Tillman (1962) are calculated according to the method given in Appendix 1.B. These figures are based on cellulose digested and are therefore not strictly accurate since VFA arises from other sources and not all cellulose digested is reflected in VFA production. The rate of 0.47 m equ/100ml/h for the system of Slyter et al. (1964) cannot be properly evaluated as the authors do not make clear whether the VFA taken up by the dialysis system was accounted for in calculating the quoted figure. A preceding paper from the same group of workers (Rufener

Table 9 Comparative Molar Proportions of Volatile Fatty Acids (VFA) in vitro and in vivo.

No.	Reference		Molar Proportions of VFA (mol/100 mol) Acetic acid	Propionic acid	Butyric acid	Notes
3	Davey, Cheeseman and Briggs, 1960	in vitro	46.4	29.7	22.2	other acids : 3.5
		in vivo*	46.0	22.9	21.5	other acids : 9.6
6	Gray, Weller, Pilgrim and Jones, 1962	in vitro	68.3	17.7	13.9	means of 3 experiments
		in vivo†	68.5	17.8	13.6	
9	Aafjes and Nijhof, 1967	in vitro	72.4±8.6	28.4±4.1	11.7±2.1	mean of 22 samples
		in vivo	78.6±6.8	20.5±4.2	10.6±1.3	mean of 2 cows

*Control cow, same diet

†Sheep

Table 10 Production rates of Volatile Fatty Acids (VFA) in vitro

	Reference	Type	TVFA rate m.equ./100ml/h	Notes
1	Warner 1956	P[1],B short	0.195	Calculated from given cellulose digestion data. Assumes 65:20:15 fermentation pattern
3	Davey, Cheeseman and Briggs 1960	P[1],P long	0.184 Culture vol. basis 0.292 Substrate flow basis	Calculated from graphical data - total figures including dialysed fraction
4	Stewart, Warner and Seeler 1961	I,C,SS short	1.3	Authors figure; allowance made for dilution rate
5	Harbers and Tillman 1962	I,C,SS short	0.054-0.2 Culture vol. basis 0.177-1.611 Substrate flow basis	Calculated from given cellulose digestion data. Assumes 65:20:15 fermentation pattern
7	Rufener, Nelson and Wolin 1963	P[1],P,F long	0.75-0.87	"Apparent" total rate, including dialysed fraction
8	Slyter, Nelson and Wolin 1964	P[1]P,F long	0.47	VFA in dialysate may not be included - see text
10	Czerkawski and Breckenridge 1969	I,B short	0.37-1.2	Authors figure
	Carrol and Hungate 1954	RUMEN	1.42-2.35	Typical in vivo figures

P[1] = permeable, F = flow through, I = impermeable, B = batch, C = continuous, P = periodic feed, SS = steady state, short/long = duration

et al. 1963) does state that dialysed VFA's were included in the quoted production rate. The figures for the latter case (0.75 to 0.87 m equ/100ml/h) are approximately double those for the former, suggesting that the dialysed VFAs were ignored by Slyter et al. (1964) in reaching the figure of 0.47 quoted. A further uncertainty in these figures arises from the effects of washout in continuous systems, whether periodic or steady state. Some of the VFA's produced are "lost" when artificial saliva or substrate is added to the culture vessel and displaces its volume from the effluent overflow. Rufener et al. (1963) described their figure for VFA production as "apparent", implying that it did not take wash out into account. Accurate quantification of VFA production is possible with in vitro systems since additions to and removals from the culture can be accurately monitored. Few of the authors cited have taken full advantage of this and much of the potential of their experimental work remains unexploited. The results shown in Table 10 must therefore be treated with caution since, apart from their different derivations, their quantitative accuracy may have been reduced by failure to take account of certain experimental factors, such as actual (rather than apparent) rates of dilution.

It appears that impermeable systems (Table 10/2, 4, 5 and 10) give rise to higher rates of VFA production than their permeable counterparts (Table 10/1, 3, 7 and 8) and that rates measured in systems where culture

periods are short (Table 10/1, 5 and 10) are higher than in those with extended culture periods (Table 10/3, 4, 7 and 8). The observation of higher rates in short-term cultures is consistent with the common finding that numbers and activity of rumen organisms tend to diminish in long term in vitro culture and stabilise at lower than in vivo levels. With the exception of Warner (1956), the permeable long-term culture systems displayed low rates of VFA production, which probably reflected the culture duration rather than permeability.

3. OBJECTS OF STUDY.

OBJECTS OF STUDY

In view of the widespread and increasing use of antimicrobial compounds as silage additives, it was decided to carry out experiments to assess the effects of these compounds in the rumen.

In order to make such an assessment it was proposed to examine a number of silages made with the additives discussed in the literature review; ie. formic acid, acetic acid and formaldehyde. Two experimental approaches were planned; 1) in vivo experiments using rumen-fistulated sheep, and 2) in vitro experiments using an in vitro rumen, the design of which should be derived from consideration of those discussed in the literature review.

An investigation of the effects of additive compounds *per se* was regarded as a necessary part of this work and it was proposed to carry this out by infusing pure additives into in vitro cultures.

SECTIONS 4 - 8. EXPERIMENTAL.

4. ANALYTICAL METHODS.

A. CHEMICAL

1. AMMONIA Ammonia concentrations were determined in 5 ml samples of rumen liquor, using the microdiffusion technique of Conway and O'Malley as described by Chalmers, Cuthbertson and Synge (1954) with modifications suggested by Chalmers (1968). The procedure is detailed in Appendix 1.E.

2. VOLATILE FATTY ACIDS 8 ml samples of rumen liquor, and dialysate were cooled to 0-4 ^{o}C and deproteinised as described by Packett and McCune (1965). Estimates of VFA concentration were made by gas chromatography (GLC) using direct injections of the acidified deproteinised samples.

A solution containing known amounts of volatile fatty acids, subjected to the same deproteinisation and centrifugation as the samples, was used as standard. Generally one standard was injected for every ten rumen liquor or dialysate samples.

In order to increase the accuracy of measurement of higher acids, amplifier attenuation was decreased during their detection. This resulted in an irregular base-line, making integration measurements impossible, but since peaks were narrow and Gaussian, peak height measurements could be made and concentrations calculated by comparison with standard peak heights.

Details of GLC techniques used are given in Appendix 1.F.

3. DRY MATTER DM was determined by drying in an oven at 100° C for sixteen hours.

4. ANALYSIS OF SILAGES Determinations of DM, pH, total nitrogen (TN), total soluble nitrogen (TSN), volatile nitrogen (VN), WSC, buffering capacity (BC) and lactic acid in silages were carried out according to the methods described by McDonald, Henderson and McGregor (1968).

B. MICROBIOLOGICAL

1. CULTURAL COUNTS Anaerobic culture techniques were based on those of Bryant and Robinson (1961), Blackburn and Hobson (1962), Hungate (1966) and Latham and Sharpe (1971).

Oxygen-free gas. Commercial gas, from cylinders, was passed through a combustion tube containing copper turnings maintained at 300 - 350° C by a furnace. Details of the furnace design are given in Appendix 2.B.

Gassing jets. Jets were made by bending lengths of 1.58 mm diameter stainless-steel tubing and securing these to short glass butts. The butt allowed connection to a gas filter by means of butyl rubber tubing. Gas filters were sterilised by autoclaving and jets were stored in 95% ethanol when not in use.

Gas mixtures. Pure CO_2 was used during reduction and buffering of prepared media. A mixture of N_2 and CO_2 (9:1) was employed during manipulation of dilutions and culture tubes.

Media containers. Roll-tube cultures were made in 150 x 18 mm glass test tubes. These were routinely sterilised inverted in wire baskets lined with aluminium foil. After aseptic addition of medium, the tubes were plugged with sterile rubber bungs.

Dilutions were prepared in 25 ml vials, stoppered with 15 mm rubber bungs. Bulk quantities (100 ml) of basal media were stored in screw-cap bottles, under refrigeration.

Roll tubes. 18 mm tubes were rolled on the apparatus shown in Fig. 12. A synchronous motor rotated the tube directly, via a rubber wheel. Cooling water was constantly passed around the tube whilst it rotated to hasten gelling of the agar. Details of this machine are given in Appendix 1.G.

Reducing agents. A mixture of cysteine-HCl and sodium sulphide (0.15 g/l each, final concentration) was used as the reducing agent for both media and dilutions. Resazuring (0.001 g/l) was incorporated into the media as a redox indication.

Preparation of media. Formulae of media used for viable

counts are given in Appendix 1.H. They are, 1) a dilution fluid for serial dilutions of the sample, and 2) media for selective cultivation of rumen organisms. The cultivation media were of the rumen-fluid based type (Bryant and Robinson 1961) containing the following specific substrates as "selective agents" : 1) cellulose as sole carbohydrate source, 2) gelatin as sole organic nitrogen source, and 3) starch as sole carbohydrate source.

Media were prepared in bulk and distributed in the reduced state. All ingredients except agar and those added after sterilisation were combined and adjusted to pH 6.8 with NaOH. After adjusting pH the final volume was made up with water allowing for the addition of bicarbonate and reducing agents after autoclaving.

Distribution of media. After reduction media were distributed by pipette in 2.5 ml amounts into 18 mm tubes which were sealed with rubber bungs. Gassing jets were introduced into the bottle of medium and each tube as it was filled. Agar media were maintained in a liquid state until inoculation, then rolled and incubated.

Preparation of dilutions. Initial dilution of rumen liquor was by withdrawl of 1 ml from the centre of the sample flask, after mixing and gassing. Subsequent dilutions were made in the usual way, by successive 1 ml transfers but with gassing jets inserted into each opened vial of diluent.

Roll tubes were inoculated in triplicate with 0.5 ml from the -5, -6 and -7 dilutions of in vivo samples and -4, -5 and -6 dilutions of in vitro samples.

Incubation and counting. Tubes were incubated upright, at 39° C for 21 days. Colonies were counted by marking off with a pen connected to an electrical counter.

2. DIRECT COUNTS Direct microscopic counts of both protozoa and bacteria were made by methods based on those of Warner (1956).

Sampling and storage. Rumen liquor samples were stored deep frozen, as equal mixtures with formalised 30% glycerol.

Protozoal counts. Protozoans were counted in a 0-2 mm deep counting chamber with a 1/16 mm^2 grid. Five squares of the grid were counted for each sample and the mean count used to calculate the final value.

Bacterial counts. A counting chamber of 0.02 mm depth and 1/400 mm^2 grid was used in conjunction with a X 40 phase-constrast microscope objective. Since the depth of field of the objective was less than the depth of the chamber, careful focusing throughout the field was necessary to ensure that every cell was counted. Five squares were counted for each sample.

5. SILAGE ANALYSIS.

Three groups of experimental silages made under field conditions on the School of Agriculture farms were studied. A further silage was used as substrate for infusion experiments. The additive levels and analysis data are given in Tables 11 - 14.

Table 11. Group I silages.

Ensiled 28.8.72; Harvested from Lower Lambing Field, Boghall Farm.

Ensiled in 500 kg capacity plastolene silos.

TREATMENT	RATE OF APPLICATION (g/kg fresh wt.)	DM (g/kg)	pH	TN (g/kg DM)	TSN (g/kg DM)	VN (g/kg DM)	EtOH (g/kg DM)	WSC (g/kg DM)	BC in (m equiv/ 100g DM)
Control	-	191	4.11	27.4	15.8	1.7	4.9	38.0	118
Formic acid/ acetic acid	4.2:1.4	198	4.12	27.7	12.6	0.89	8.0	96.4	86
Formalin/ acetic acid	4.5:4.5	208	4.62	26.0	9.3	0.61	6.4	144.5	95

Formalin applied as 40% solution of formaldehyde.

Seeding mixture in Appendix 1.I

Table 12. Group II Silages.

Ensiled 18-20.7.72 in 25 Mg vacuum silos at Woodhouselea Farm.

Harvested from Jean Lowrie Field and Howgate Stackyard, Easter Howgate Farm.

TREATMENT	RATE OF APPLICATION (g/kg fresh wt.)	DM (g/kg)	pH	TN (g/kg DM)	TSN (g/kg DM)	VN (g/kg DM)	EtOH (g/kg DM)	WSC (g/kg DM)	BC in (m equiv/ 100g DM)	Lactic acid (g/kg DM)
Control	-	207	3.93	24	12.2	1.7	4.8	7.1	106	8.4
Wilted	-	314	4.18	21.7	11.8	1.6	5.9	54.9	102	8.6
Fresh + formic acid	3.6	212	3.84	25.5	13.9	1.6	38.3	71.0	79	5.0
Wilted + formic acid	6.3	316	4.17	23.2	10.5	1.2	3.8	193	60	7.0

Formic acid applied as 'Add-F'

Seeding mixture in Appendix 1.I

Table 13. Group III Silages.

Ensiled 23.8.72 in 2 Mg plastolene silos.

Harvested from Lower Lambing Field, Boghall Farm.

TREATMENT	RATE OF APPLICATION (g/kg fresh wt.)	DM (g/kg)	pH	TN (g/kg DM)	TSN (g/kg DM)	VN (g/kg DM)	WSC (g/kg DM)	BC in (m equiv/ 100g DM)	Lactic acid (g/kg DM)
Control	-	213	4.1	30.5	17.7	2.6	41.0	96	65
Formalin/ formic acid	8.2:2.1	212	5.05	31.3	8.7	1.1	133	56	38
Formalin/ H_2SO_4	5.25:1.75	218	3.98	32.1	14.1	2.0	64.0	82	106
Formalin	9.7	213	4.90	35.0	11.8	6.0	151	64	16

Formalin applied as 40% solution of formaldehyde.

H_2SO_4 applied as a 20% solution.

Seeding mixture in Appendix 1.I

Table 14. S37 Silage.

Ensiled 24.7.73 in 3 Mg plastolene silos.

Harvested from Hayknowes Field, Boghall Farm.

TREATMENT	DM (g/kg)	pH	TN (g/kg DM)	TSN (g/kg DM)	VN (g/kg DM)	EtOH (g/kg DM)	WSC (g/kg DM)	Lactic acid (g/kg DM)
Wilted	255	4.12	19.9	12.3	1.7	8.4	3.1	72

Seeding mixture in Appendix 1.I

6. IN VIVO EXPERIMENTS.

INTRODUCTION

In vivo experiments were carried out with sheep in metabolism trials and were primarily concerned with the effects of additive treated silages on the rumen microflora. Chemical measurements made on rumen liquor samples from the same trials will be reviewed in the discussion. The silages were also studied in vitro and results of these experiments are presented in Section 7.

EXPERIMENTAL MATERIALS & METHODS.

ANIMALS. Mature Cheviot wethers fitted with permanent rumen canulae similar to those of Alexander (1970) were used.

SILAGES. More than enough silage for a trial was taken at one time from a given source and thoroughly mixed. The silage was bagged in amounts sufficient for one meal and stored at - 20°C, and removed to room temperature 24 to 36 hours before feeding.

TRIAL PROCEDURE. The trial with Group I silages used a single experimental period during which each silage was fed to a group of three sheep. With Groups II and III, eight sheep were used in a Latin

square, cross-over design balanced for residual effects. Each sheep was fed each silage.

Techniques employed were the same for all experiments. Seven to fourteen days prior to commencement of a trial the sheep were introduced to a silage of different composition to the experimental materials. At the end of this introductory period the sheep were placed in metabolism crates (McDonald 1958). The animals were allowed 2 to 3 days to adapt to the crates before being offered the experimental rations.

The silages were given in equal feeds at 09.00 and 21.00 daily and residues removed at 11.00 and 23.00 respectively. Experimental diets were introduced gradually and after seven days DM intakes were recorded at each feed,over an eight day period. Oven DM determinations were carried out on the residues from each sheep, the true DM intake being calculated by applying a factor based on DM determination by toluene distillation (Dewar and McDonald 1961).

With the Group II and Group III silages a changeover period of three or four days, during which the new diet gradually replaced the old, was allowed before each experimental period.

Water was available to the sheep at all times

during the trials and fresh water was offered at the same time as the food. Maintenance requirements of minerals and vitamins (Agricultural Research Council 1965) were met by a supplement given with each feed.

SAMPLING. After a minimum of fourteen days on a diet the rumen contents were sampled at 11.00 h (ie 2 h post feeding). Rumen contents were withdrawn into a Dewar flask which had previously been warmed to 40°C with water. The sample was taken from several regions of the rumen by repeated insertion and withdrawl of the sampling tube. Samples were mixed and the initial dilution made as soon as possible, but always within 1 h of removal.

RESULTS.

GROUP 1 SILAGES.

Compositions of Group 1 silages are given in Table 11 (page 85). Table 15 gives mean differential viable counts and NH_3-N values on rumen liquor obtained with sheep offered these silages.

Differences between the control and the formic acid/acetic acid-treated silages were not significant in all cases. Counts on the starch medium for the formaldehyde/acetic acid-treated silages did not differ significantly from those for the other materials but were significantly lower on gelatin and higher on cellulose media. Ruminal NH_3-N was also significantly lower with the formaldehyde/acetic acid-treated silage.

Table 15. Log mean differential viable counts (per ml), and NH_3-N (mg/l) in rumen liquor from sheep given Group 1 silages.

	silage		
	control	formic acid/ acetic acid	formaldehyde/ acetic acid
starch medium	6.22a	5.78a	6.42a
gelatin medium	6.59a	6.54a	6.22b
cellulose medium	5.14a	5.57a	6.37b
NH_3-N	356a	379a	268b

a,b; means without the same letter differ significantly ($P < 0.01$)

GROUP II SILAGES

Compositions of Group II silages are given in Table 12 (page 86). Table 16 gives mean differential viable counts in rumen liquors of sheep offered these silages.

Analysis of variance indicated that DM intake had a significant effect on the counts. Analysis of covariance was therefore carried out, with DM intake as covariate. The wilted, formic acid-treated material gave rise to significantly higher counts on starch and gelatin medium, compared with the other silages. There were no significant differences between any of the silages in the case of cellulose medium.

Table 16. Log mean differential viable counts (per ml) in rumen liquor from sheep given Group II silages.

	silage			
	fresh	wilted	fresh + formic acid	wilted + formic acid
starch medium	5.20a	5.24a	5.28a	5.51b
gelatin medium	5.42a	5.31a	5.60a	6.01b
cellulose medium	5.36a	5.16a	5.31a	5.54a

a,b; means without the same letter differ significantly ($P < 0.01$)

GROUP III SILAGES.

Compositions of Group III silages are given in Table 13 (page 87). Table 17 gives mean differential counts in rumen liquors of sheep offered these silages.

Analysis of variance indicated that DM intake had a significant effect on the counts. Analysis of covariance was therefore carried out, with DM intake as covariate.

The highest count on the starch medium was obtained with sheep offered the formaldehdye/H_2SO_4-treated silage, this being the only value differing significantly. On gelatin medium no significant differences between the silages were apparent. The control silage gave lower counts of cellulose-utilising organisms than the others.

Table 17. Log mean differential viable counts (per ml) in rumen liquor from sheep given Group III silages.

	silage			
	control	f/dehyde formic acid	f/dehyde H_2SO_4	f/dehyde
starch medium	5.51a	6.00a	6.16a	5.45a
gelatin medium	5.94a	5.32a	5.85a	5.40a
cellulose medium	5.26a	6.02b	6.15b	6.03b

a,b; means without the same letter differ significantly ($P < 0.01$)

(f/dehyde = formaldehdye)

DISCUSSION

Data additonal to those given in the results section and relevant to this discussion, were obtained by Edwards 1976 and are summarised below.

Additional data from sheep metabolism trials with Groups I, II and III silages.

Group	Silage	DM intake (g/d)	NH_3-N (mg/l) 2h post-feeding	TVFA (mM/l) 2h post-feeding
I	control	924	356	-
	formic acid/ acetic acid	989	379	-
	formaldehyde/ acetic acid	1008	268	-
II	control	697	300	-
	wilted	864	286	-
	fresh/ formic acid	711	323	-
	wilted/ formic acid	883	272	-
III	control	904	438	108
	formaldehdye/ formic acid	1001	303	108
	formaldehdye/ H_2SO_4	985	431	108
	formaldehyde	1008	312	114

Most of the significant differences in ruminal microbial counts arose with the Group I and Group III formaldehdye-treated silages. With the Group I silages, formaldehyde treatment resulted in reduced numbers of proteolytic organisms in the rumen compared with the other

treatments, while the cellulose digesters were present in larger numbers. This latter observation was confirmed with the Group III silages. Counts on cellulose media were positively correlated with DM intake in both groups ($r = 0.89$ and 0.94). The highest DM intakes were achieved with the additive treated silages, in which fermentation in the silo had been restricted. This restriction was reflected in high residual WSC (Tables 11 and 13) and, in general, its magnitude was related to total levels of additive application, regardless of type (see details overleaf). It may be that the differences in counts of ruminal cellulolytic organisms were a reflection of differences in DM intake and not a result of the presence of formaldehyde per se.

The use of formaldehyde in silage making can have variable effects on intake. Wilins, Wilson and Cook (1973) found that intakes were improved significantly by application rates of 3-5 g/kg of fresh grass but reduced by rates in excess of 5.5 g/kg. Valentine and Brown (1973) showed that the use of 2.6 g of formaldehyde per kg of lucerne during ensilage improved intake, but not significantly. Use of an additional 5 g/kg formic acid resulted in a significant increase in intake. Formaldehyde additions of 9.03, 13.55 and 18.02 g/kg during ensilage of lucerne were found by Brown and Valentine (1972) to depress intake significantly. This dualistic effect of formaldehyde

Relationship of silage WSC to DM intake
and to total additive application rate.

Silage Group	WSC (mg/kg DM) [WSC]	DMI (g/d) [DMI]	total application rate (g/kg) [AR]
I	38.0	924	0
	96.4	989	5.6
	144.5	1008	9.0
II	7.1	697	0
	54.9	864	0(W)
	71.0	711	3.6
	193	883	6.3(W)
III	41	904	0
	133	1001	10.3
	64.0	985	7.0
	151	1008	9.7

(1) $WSC = 17.24\ AR + 0.003$

$r = 0.8069$

$p < 0.01$

(2) $DMI = 815.8 + 1.006\ WSC$

ie. $DMI = 816 + WSC$

$r = 0.5156$

$p < 0.1$

is due to the fact that at lower application rates the formaldehyde will be wholly taken up by forage protein whilst at higher levels there will be some residual formaldehyde which will bring about inhibition of the rumen microflora (Wilkins et al. 1973). The quantity of free formaldehyde in the silage will clearly be a function of crop protein content and application rate. The silages in the present experiments were all treated with < 5g formaldehyde per kg fresh herbage, a level at which the inhibitory effect on the rumen microflora would be absent and DMI improved.

There was a high correlation between ruminal NH_3-N and microbial counts on the gelatin medium ($r = 0.95$), both being lower in the case of formaldehyde treatment. The protection of forage protein against microbial attack, brought about by the protein-binding effect of formaldehyde, was almost certainly the cause of these differences.

Rumen microbial counts with Group II silages were highest, on all three selective media, with the formic acid-treated wilted silage (Table 16). The difference was less significant with the cellulose medium ($p < 0.05$) than the other media ($p < 0.01$). The high residual WSC of the formic acid-treated wilted silage (193 mg/kg DM) is a consequence of restricted fermentation due in part to wilting and in part to additive treatment.

Both the wilted, untreated material and the treated fresh material had lower WSC contents owing to a greater degree of fermentation. The less fermented silages also had higher DM intakes and this contributed to the higher microbial counts obtained with these materials. The relationship between increased silage DM and increased intake is well established (Thomas, Moore, Okamoto and Sykes, 1961 and Jackson and Forbes, 1970). This is apparently unrelated to moisture content per se. (McDonald 1975) but Donaldson and Edwards (1976) have suggested that it may be due to restriction of fermentation in the silo. This does not appear to be the case here since the formic acid treated unwilted silage in Group II had more WSC than the wilted and was apparently less fermented.

The lowest ruminal NH_3-N concentrations with Group II were measured with the formic acid-treated wilted silage. This indicates that the ruminal microflora were not "energy limited" which has been suggested as being the case with silage diets (Durand et al. 1968, Conrad and Hibbs 1968), or that the nitrogen fraction of this silage was less easily degraded or more readily assimilated. The figures given on page 100 indicate that the nitrogen fraction of the formic acid-treated wilted silage contained a low proportion of NPN, which lends support to the contention that it was less degradable. There is a

Group II silage	silage: TN (g/kg DM)	silage: NPN (g/kg DM)	silage: proportion NPN : TN	ruminal NH_3-N (mg/l)
control	24	13.9	0.58	300
wilted	21.7	13.5	0.62	286
fresh/ formic acid	25.7	15.5	0.61	323
wilted/ formic acid	23.2	11.7	0.50	270

possible further explanation for the low ruminal ammonia-level in terms of the ease with which certain fractions could be absorbed from the rumen, but this is rather speculative.

On the basis of NPN content the ruminal NH_3-N concentration with the wilted silage was anomalous in being the next lowest, since this silage had a higher NPN content than the control, which gave a higher ruminal ammonia. The greater supply of readily available energy in the form of the higher WSC content of the wilted silage may partly explain this. The complexity of the situation is emphasised when one considers that the higher WSC of the formic acid-treated unwilted material which contained similar NPN, was not associated with a low ruminal ammonia concentration.

The Group II silages also yielded a positive

correlation between WSC content and counts on starch medium ($r = 0.99$ and $p < 0.01$). It is tempting to suggest that this may have been due to a selective effect of high WSC on organisms adapted to the utilisation of soluble substrates, but such a relationship was not evident with the Group I and Group III silages. In these groups none of the silages were wilted and some of the treatments included formaldehyde.

The most consistently obvious relationship in the present data was that between the extent of fermentation and DM intake. Silage WSC and DM intake were the only parameters to show high positive correlation coefficients for all groups of silages. The influence of additives in this series of experiments was mediated through their effects a) in providing different substrates for the rumen and b) their effect on dry matter intake. There is no evidence that additives themselves had affected rumen function.

7. IN VITRO EXPERIMENTS.

INTRODUCTION

Adequate precedent for the application of in vitro techniques to the study of rumen microbiology and biochemistry has been established in the literature review. Certain definable approaches have also been distinguished; simple "all-glass" impermeable systems, more complex semipermeable systems, batch cultures, continuous cultures and others.

The aim of the present study was to investigate the effects of silage additives on rumen metabolism and, in particular, on the microflora. It was a comparative study and required a well defined system with minimum experimental variables. Whilst simulation of the rumen in physio-chemical terms was a primary objective for the in vitro apparatus, absolute simulation was never considered feasible nor even desirable. The principle of steady state operation could therefore be adopted and the equipment was developed from the conventional chemostat.

EXPERIMENTAL MATERIALS & METHODS.

THE IN VITRO RUMEN The design criteria adopted in the development of the in vitro rumen were as follows :

1. anaerobic operation.
2. constant flow of substrate.
3. constant volume of culture.

4. control of temperature and pH.
5. facilities for dialysis of culture fluid.
6. adequate mixing of culture.

In addition it was desirable that the equipment should have facilities for:

a) measuring and recording temperature, Eh and pH.
b) using O_2-free gas to aid mixing of the culture, to maintain anaerobiosis and transport effluent.
c) storage of buffer solution, dialysate, substrate, effluent and spent dialysate.

On the basis of the criteria set out above, a theoretical design for a continuous, steady-state, in vitro rumen apparatus was evolved (Fig. 13). Performance characteristics for the various components of the equipment were next defined. It was considered desirable that the apparatus should meet the following specifications:

i. all vessels, connecting tubing and joints to be impermeable to gases.
ii. pumping of liquids to be controlled and reproducible within $\pm$ 5%.
iii. temperature control and display to be accurate within $\pm$ 0.25°C and pH control within $\pm$ 0.025 pH.
iv. culture stirring at speeds between 100 and 200 r.p.m. to be possible.
v. supply of O_2-free gas, regulated at 0.2 - 0.5 l/min to be provided.

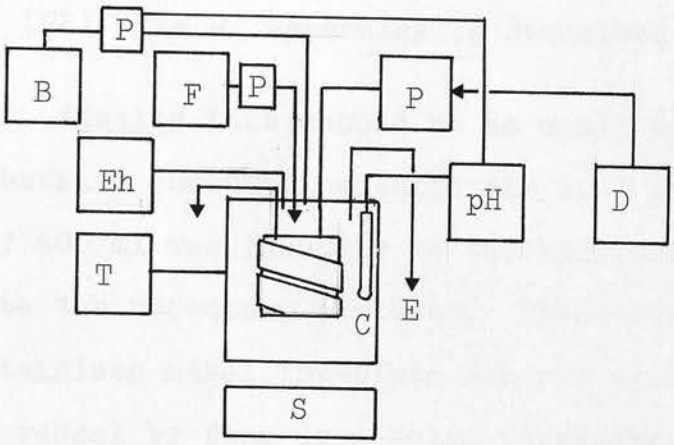


Fig. 13 Theoretical design for continuous steady-state in vitro rumen apparatus. (cf., Fig. 11, page 65)

B	Buffer	F	Feed
C	Culture	P	Pump
D	Dialysate	S	Stirrer
E	Effluent	T	Temperature control

DEVELOPMENT Construction of equipment to the above specification was carried out in two stages. During the first stage a complete working apparatus was built, but proved to be cumbersome in use and difficult to maintain. This apparatus (R1) was therefore modified to produce a second machine (R2). The R1 apparatus is described below.

CULTURE VESSEL Ideally this should be as small as possible to minimise substrate consumption (Appendix 1.A) and a gross volume of 600 ml was found to be the smallest which could accomodate the necessary fixtures. These were mounted on a stainless steel top-plate secured against the rim of the vessel by four draw bolts (Appendix 2.A). A magnetic stirrer was suspended on the draw bolts below the culture vessel. The culture vessel, the top plate and the stirrer were arranged in a frame, independent of the main equipment (Fig. 14).

MAIN FRAME The main frame was made of aluminium sheet with a bonded PVC finish and consisted of four shelves supported by uprights. The lowest shelf contained two pH meters (one for Eh measurement) and the effluent reception vessel. The second shelf was occupied by the electronics unit and the third by miniature peristaltic pumps. The upper shelf accommodated resevoirs of dialysate, buffer and antifoam solutions.

ELECTRONICS UNIT The unit incorporated a power supply circuit, control units for pH, temperature, gas flow, automatic samplimg and for the substrate and dialysate

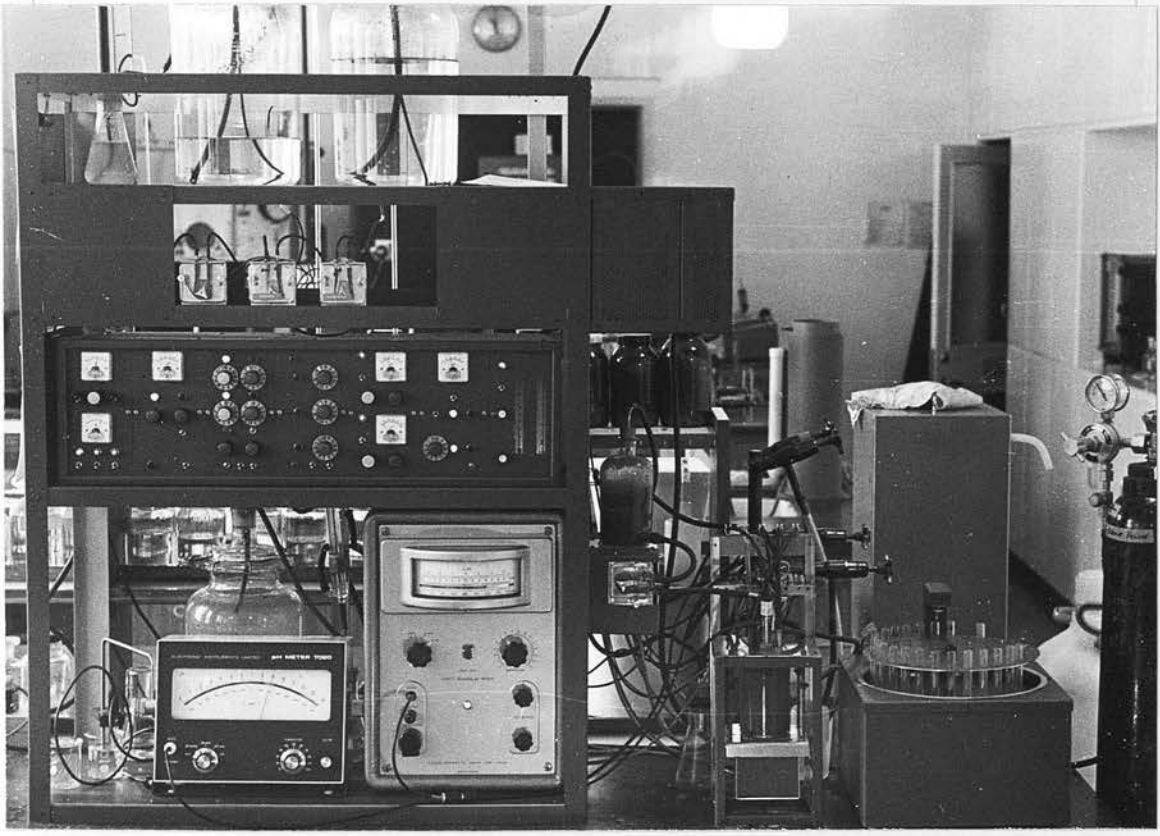


Fig. 14 The R1 apparatus - the culture vessel stands to the right of the apparatus main frame.

pumps. In the R1 version these were constructed as a single integrated assembly which limited access and made servicing difficult.

ANCILLARY EQUIPMENT CO_2 gas was supplied to the apparatus from a commercial cylinder *via* a regulator. Residual O_2 was removed by passing the gas through a combustion tube containing reduced copper at 300°C. The furnace design (Appendix 2.B) was based on that of Hobson (1971). Gas flow was controlled by pinch valves and taper-tube flow meters. The gas supply entered the culture vessel by a stainless steel tube.

An automatic sampler, arranged to sample at four hourly intervals, was constructed. The frequency of sampling was found to be excessive in comparison with the long time-constants ofa culture with a mean generation time in excess of 24h. This, together with the disturbance of culture volume brought about by sampling (partially negating the principle of steady-state working), led to deletion of the sampler in R2.

Wherever possible fluid and gas connections were made using stainless steel or glass. Where flexible sections and joints were required, butyl rubber tubing was employed.

pH MEASUREMENT AND CONTROL Combination glass/reference electrodes used routinely in laboratory pH measurement employ a small porous plug of ceramic material at the

liquid junction of the calomel half-cell. The fine pores of such plugs readily become blocked by colloids when immersed in rumen contents. The addition of pressure-balancing pipes, to prevent a net flow into the liquid junction, did not overcome this problem. A remote liquid junction, using a separate reference electrode linked by saturated KCl/agar-saturated KCl, was found to function satisfactorily (Appendix 2.C).

pH adjustment was arranged to take place in response to a fall in culture pH, using artificial saliva as the control agent. Two problems were associated with the formulation of this solution. The first was that the dry matter content of the substrate was 4% compared with typical ruminal values of around 14% (Makela 1956). To avoid further reductions in DM, an artificial saliva of approximately 4 x natural concentration was used. This was based on that of McDougall (1948) and had the following composition :

$NaHCO_3$	49	
NaCl	2.4	
Na_2HPO_4	18.5	
$(NH_2)_2CO$	1.0	
KCl	2.8	
$MgSO_4 7H_2O$	0.6	(g/l)

Early experiments indicated that, delivered in response to pH changes, this solution constituted some 25% of total fluid deliveries to the culture vessel.

The second was that of culture fluid osmolality, which is important because of the sensitivity of rumen micro-organisms (Heald and Oxford 1953). The significance of this was recognised by Quinn (1962) whose continuous (pure) culture apparatus incorporated automatic control of salinity. The accumulation or depletion of ions, or gross changes in osmolality, could have a detrimental effect on an in vitro culture. The ionic concentrations resulting from 4 x dilution of the artificial saliva solution are compared with those of the solution of McDougall (1948) in Table 18.

Table 18. Concentrations of ions in artificial saliva solutions (mM/l).

	McDougall (1948)	Artificial Saliva (÷4)
Na^+	176	220
K^+	7.67	9.53
Cl^-	17.77	16.68
Mg^{++}	0.62	0.62
SO_4^{--}	-	0.60
HCO_3^-	116.7	145.8
HPO_4^{--}	25.94	32.59

The inclusion of a dialysis system may also be regarded as essential, contributing to the maintenance of stable osmalality, since an osmotically balanced dialysing solution should act as a buffer against changes in concentration.

Eh MEASUREMENT Measurement of Eh employed an inert metal (platimum) electrode immersed in the culture, and sharing the reference electrode half-cell of the pH measurement system.

DIALYSIS SYSTEM Regenerated cellulose and related materials have long been used as semipermeable membranes for laboratory dialysis, usually in the form of "Visking" tubing. Materials derived from cellulose might not be expected to survive exposure to the rumen environment, but most permeable in vitro systems have successfully employed this material (Table 7/1, 3, 6, 7 and 8).

Alternative membranes, based on synthetic co-polymers, have recently been developed for renal dialysis. During development of R1 various synthetic membranes were evaluated.

Dialysis membranes must possess a balance between water sensitivity and wet strength. They must wet sufficiently well to permit transport, but resist dissolution and rupture by water. In synthetic membranes this property is achieved by co-polymerisation, one polymer providing strength, the other sensitivity (Courtney 1973).

The synthetic co-polymers which were evaluated in vitro, and the results of these trials, are given in Table 19. A membrane of acrylonitrile-acrylic acid, modified by treatment with ethylene oxide, gave the best

Table 19. Synthetic membranes evaluated in vitro.

Membrane copolymer			
Support polymer	Sensitive polymer	Treatment*	Consequences of use in vitro
Acrylonitrile	dimethylaminoethyl methacrylate	U	Progressive blackening; changes similar to effects of thermal degradation †
		M	Discolouration (as above)
Acrylonitrile	acrylic acid	U	Disintegration
		M	Virtually unaltered
n-butyl methacrylate	acrylic acid	-	Embrittled

* U = unmodified

M = modified by treatment with ethylene oxide (Courtney and Gilchrist 1975)

† Identified, using infrared spectroscopy, by a peak of absorption at 1590 cm^{-1}

results and showed least evidence of damage. A further useful feature of this material is the possibility of heat sealing. Experimental membranes made from this material proved to be unsatisfactory, because of poor bonding to the rubber tubing connectors.

CELLULOSE BASED MEMBRANES The permeability of cellulose membranes is thought to be due to a sieving mechanism. Solute transport rates are directly related to pore size and film thickness, there being no charge or adsorption properties to influence transport and permeability, which are proportional to the molecular volume of the diffusing solute (Craig and Pulley 1962; Allen, Courtney, Gray, Klinkmann and Muir 1968).

Preliminary trials with cellulose membranes were encouraging. In most cases membranes survived for periods in excess of 200h without failure and diffusion rates were maintained. The resistance of cellulose membranes to cellulolysis may be explained in a number of ways. Manufactured cellulose membranes have an ordered molecular structure, which accounts for their evident transparency, and this could preclude enzymic attack by reducing the number of vulnerable "sites". Also, the hydrolysis of cellulose by the rumen microflora is known to depend upon cell adsorption onto the substrate (Grosskopf 1964), since the most efficient cellulases are insoluble. In a stirred fermenter where adhesion to the membrane is prevented, microbial attack is therefore unlikely to occur.

In all the experimental work reported here, regenerated cellulose was used as the dialysing membrane. A tube of this material was mounted as a coil on a plastic-mesh support within the culture vessel (Appendix 2.D). The membrane was replaced prior to each experiment.

Membrane area was 150 cm^2, corresponding to a surface/volume ratio of 366 cm^2/l of culture. Whilst comparisons with in vivo figures must be made with caution, particularly since the mechanisms for diffusion/transport are dissimilar, this figure appears adequate compared with a 30 l† sheep rumen (ratio 150 cm^2/l*) or that of a steer (180 l†, ratio 80 cm^2/l), even allowing for the epithelial papillae.

DIALYSIS SOLUTION. The dialysis solution was developed from the artificial saliva already described. It was desirable that the solution should be isotonic with the culture solution and have the same pH. Sodium bicarbonate was thus replaced by sodium chloride.

Table 20. Formula of dialysis solution (g/l).

NaCl	7.25
Na_2HPO_4	3.75
$CO(NH_2)_2$	0.25
KCl	0.5
$MgSO_4 7H_2O$	0.125
	pH adjusted to 6.5

* Calculated from volume assuming a spherical vessel

† Volumes quoted by Barnett and Reid (1961)

DIALYSATE DELIVERY A peristaltic pump, controlled by a method described in Appendix 2.H delivered a regular flow of dialysate through the membrane assembly. The physical continuity of flow was broken by drip chambers just before and after passage through the coil assembly (Appendix 2.D). These flow-breaks ensured that diffused solutes could not permeate back to the reservoir and contaminate the fresh dialysate, or forward through the waste pipe.

SUBSTRATE PREPARATION. Smooth and continuous delivery of substrate was necessary to fulfil the steady-state concept. Silage, the intended substrate for all the experimental work, is inherently heterogeneous and thereby incompatible with the above requirement. Accurate additions of silage, of consistent quality, could only be achieved if it was first reduced to a slurry-like liquid by mechanical homogenisation. Conventional homogenisers working by impact shear, are unsatisfactory for materials with a high fibre content. A double-shear homogeniser developed specifically for use with silage (Alexander 1969) was adopted for slurry preparation (Fig. 15).

Reliable pumping of the substrate remained a problem, the DM content of the material being of particular importance. The finely-divided particles of fibre tended to move less rapidly along the substrate-delivery pipes than the liquid portion of the slurry, resulting in blockages. This problem was overcome by the addition of a small amount (0.025%) of high substitution

Fig. 15 Double-shear homogeniser as devised by Alexander (1969).

methyl cellulose which increased the cohesion of the liquid phase, increased viscosity and lubricated the delivery pipes and pump element tube. The final DM of the slurry was adjusted to 4% and the pH to 3.50. They were thus stable when stored in a refrigerator in sealed containers. Complete details of slurry preparation are given in Appendix 2.M.

THE R2 APPARATUS The R2 equipment was much more compact than R1 and layout was more logical (Fig. 16). A shelf in front of the main-frame supported the culture vessel between two pairs of peristaltic pumps. Reservoirs for dialysing solution, buffer and antifoam were placed, together with the effluent reception vessel, on the main-frame base. Above the culture vessel, a panel carried pH and Eh meters (Appendix 2.G). Magnetic stirrers for culture and feed were constructed to a novel design using belt drive to provide increased torque at low stirring speeds (Appendix 2.E). The substrate reservoir was mounted above the left-hand pump housing which, in turn enclosed a magnetic stirrer. Gas regulation and furnace controls were fitted to a panel above the right-hand pump housing, which itself enclosed a voltage-dropping resistor for the culture heater. The furnace for O_2 removal was mounted, with suitable heat-shielding, on the rear of the main-frame. A gas-flow diagram is given in Fig. 17.

The upper main-frame was occupied by control electronics arranged as 10 "plug-in" modules (Appendix 2.H-K).

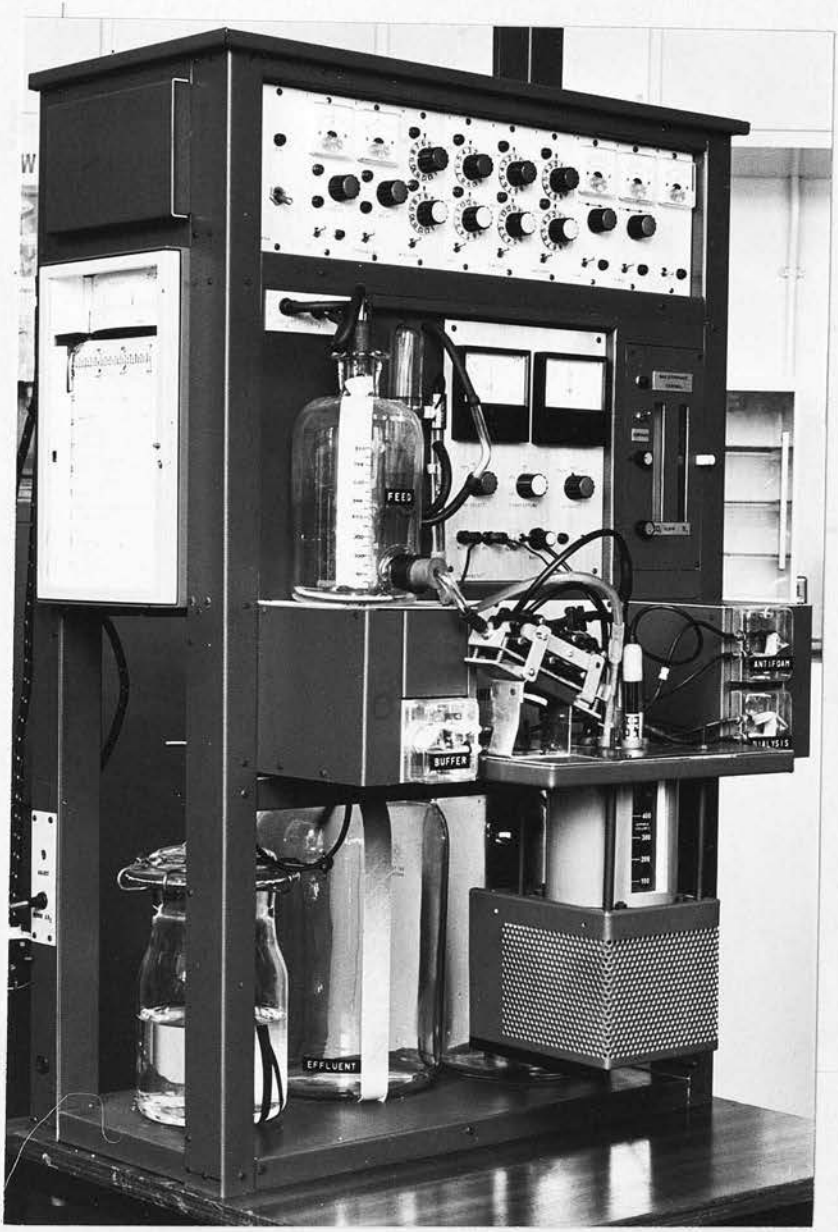


Fig. 16 The R2 apparatus.

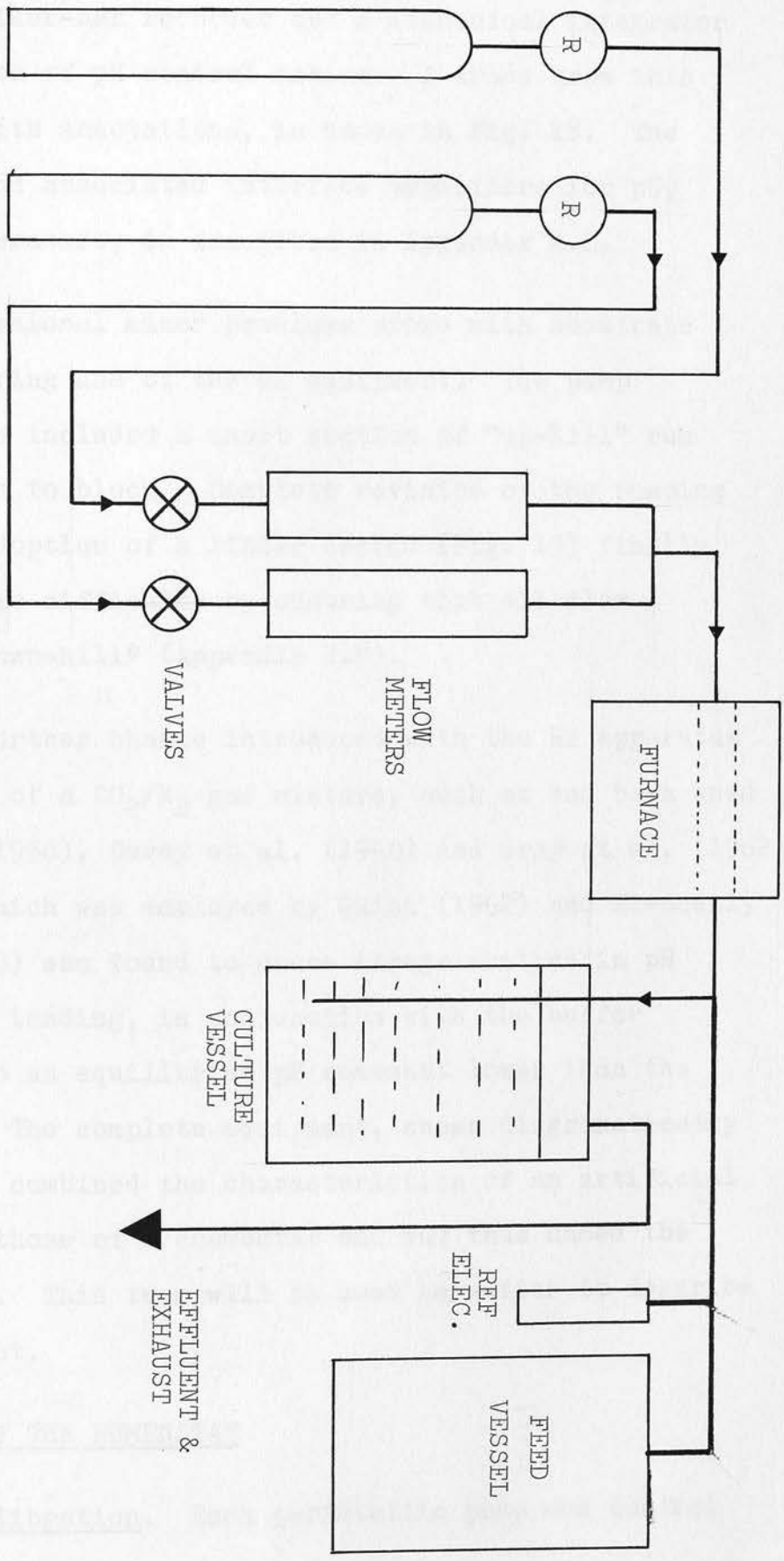


Fig. 17 Gas flow diagram for R2 apparatus.

Other components mounted on the main-frame were a six channel striker-bar recorder and a mechanical integrator for summation of pH control action. A trace from this recorder, with annotations, is shown in Fig. 18. The recorder, and associated interface amplifiers for pH, Eh and temperature, is described in Appendix 2.L.

Occasional minor problems arose with substrate delivery during use of the R2 equipment. The pump element tube included a short section of "up-hill" run which tended to block. Complete revision of the pumping system by adoption of a linear design (Fig. 19) finally resolved this difficulty by ensuring that all flow occurred "down-hill" (Appendix 2.F).

A further change introduced with the R2 apparatus was the use of a CO_2/N_2 gas mixture, such as had been used by Warner (1956), Davey et al. (1960) and Gray et al. (1962). Pure CO_2, which was employed by Quinn (1962) and El-Shazly et al. (1960) was found to cause irregularities in pH control, by tending, in conjunction with the buffer solution, to an equilibrium pH somewhat lower than the set point. The complete equipment, shown diagramatically in Fig. 20, combined the characteristics of an artificial rumen with those of a chemostat and was thus named the "Rumenstat". This term will be used hereafter to describe the equipment.

OPERATION OF THE RUMENSTAT

a) Pump calibration. Each peristaltic pump and control

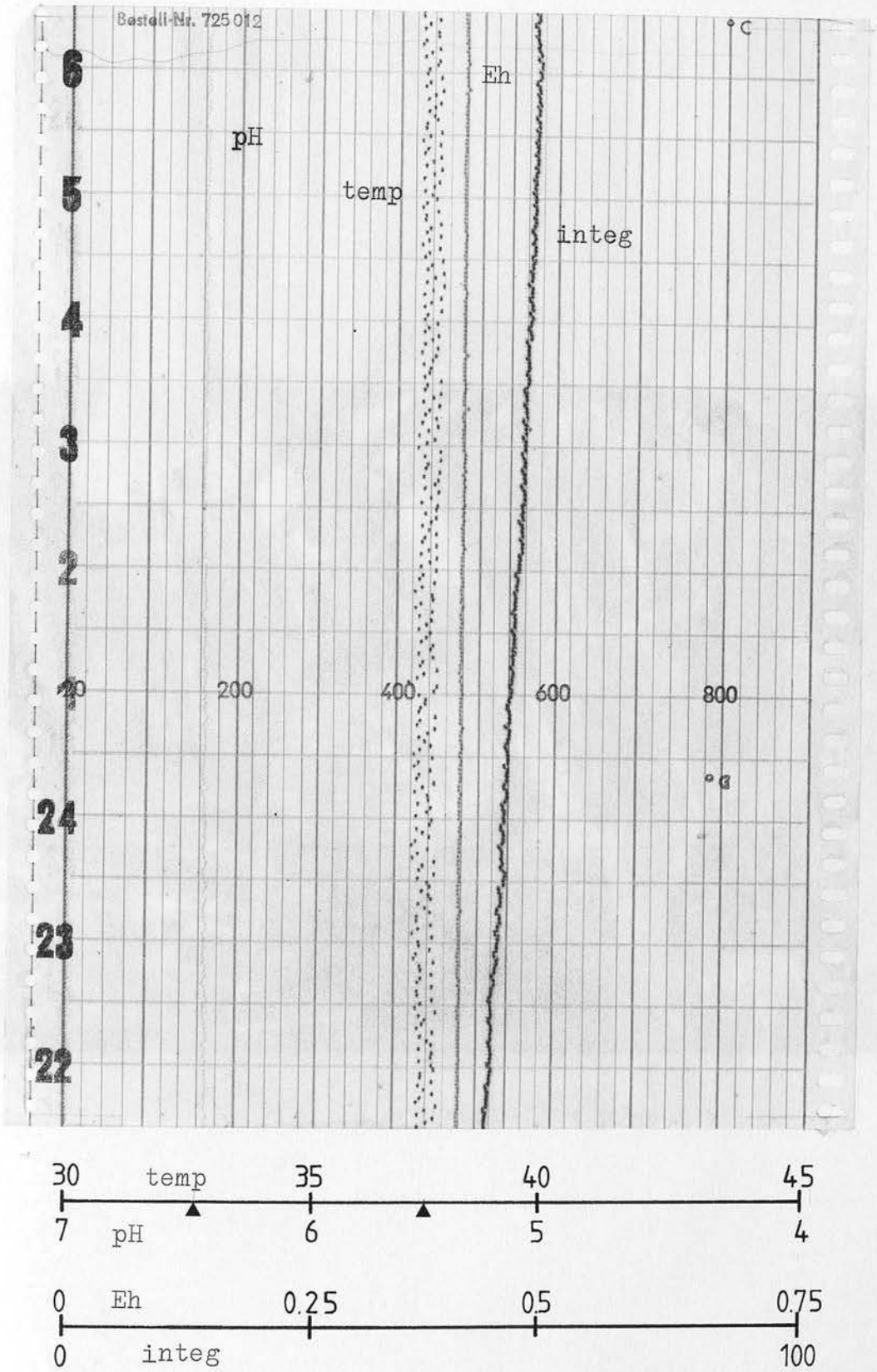


Fig. 18 Example trace from chart recorder of R2 apparatus.

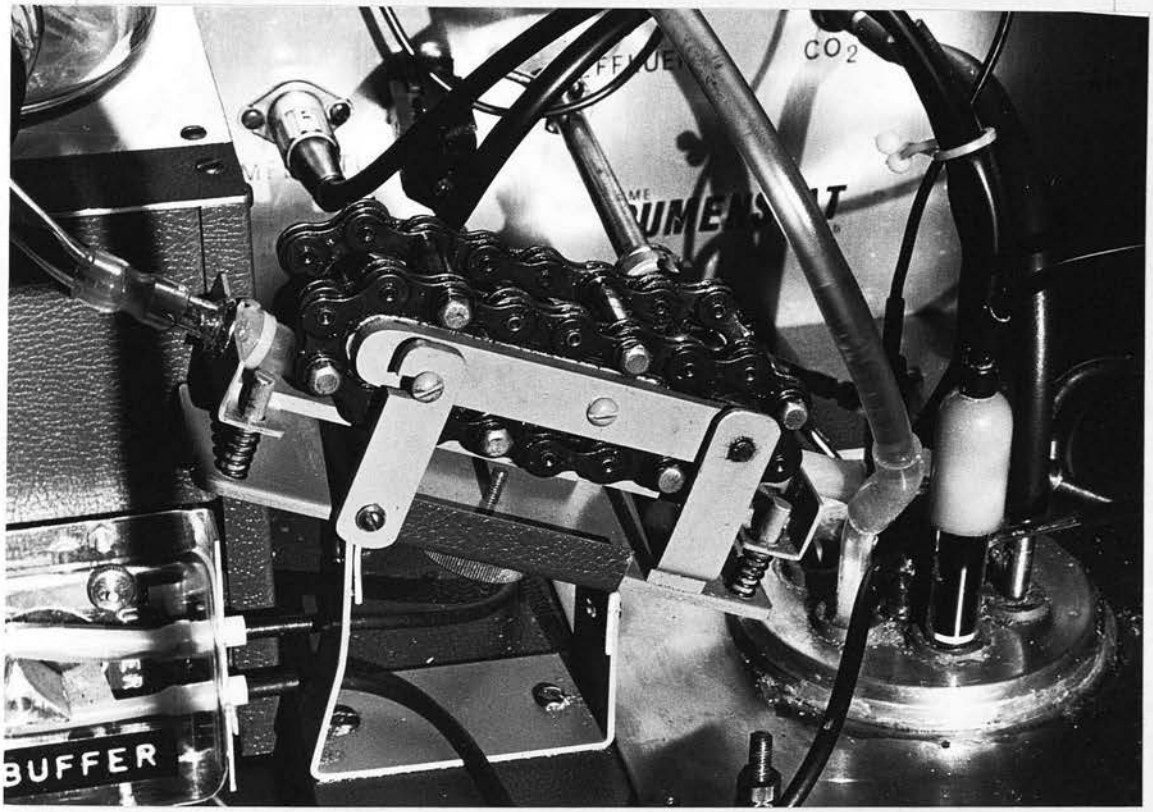


Fig. 19 Linear-flow pump.

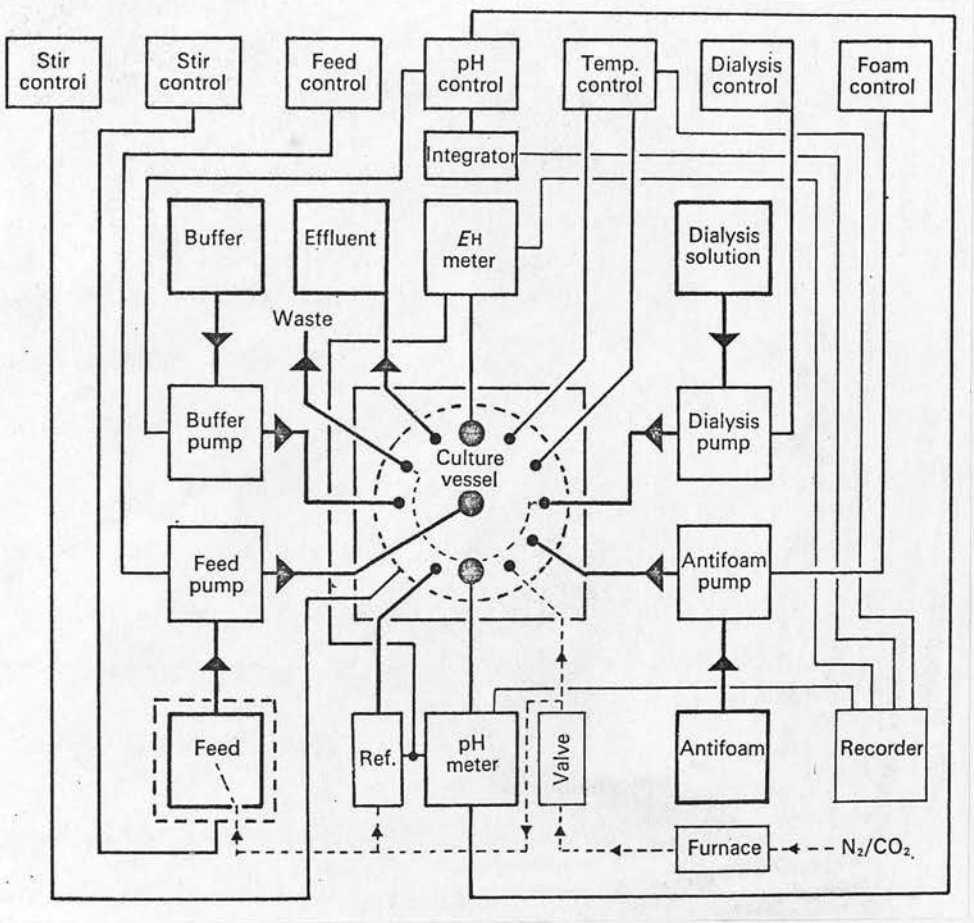


Fig. 20 Diagram of R2 apparatus.

(From Ewart, 1973)

unit combination was calibrated volumetrically for the "working" range of flow rates. Reservoir vessels were graduated and a record of daily and total deliveries was made to serve as a check on pump performance. For each experimental period pump element tubes were replaced and run for several hours before use to ensure accurate and consistent delivery.

b) Flow rates. The selection of flow rates (ml/h) for substrate (F_s), antifoam (F_a) and dialysate (F_d) had to be made in the absence of precedent. Turnover rate (T_r) (the proportion of the total culture volume in ml (V) passed through the culture vessel in 24h) was a function of substrate, buffer and antifoam deliveries to the culture. Deliveries of substrate and antifoam could be predetermined, within the accuracy of the pumping equipment. Delivery rate of buffer, whilst unpredictable could be established, within limits, on the basis of preliminary experiments.

Stewart et al. (1961) and Rufener et al. (1963) used T_r values of 2.21 and 1.43 respectively for their in vitro cultures. T_r values ranging from 0.42 to 4.80 (mean of 7 values = 1.45) were employed by Harbers and Tilman (1962). These rates seem high when compared with values obtained in vivo, which are generally less than unity (Table 21). By selecting values of 10 and 1 for F_s and F_a respectively and assuming a flow rate for buffer (F_b) of 4 an overall flow rate (F) of 15 was arrived at.

Table 21. Calculations of turnover based on lignin as marker.

Reference	Animals	Turnover rates : based on food lignin	Turnover rates : based on faecal lignin
Badawy et al. (1958 a,b)	Sheep	0.59 - 0.84	0.49 - 0.65
Rogerson (1958)	Sheep	0.38 - 0.96	0.49 - 0.97
Makela (1956)	Cows	0.39 - 0.63	-

With V = 410, T_r assumes the value of 0.88 giving good aggreement with the in vitro data in Table 21.

The rate of dialysate flow (F_d) could not be derived by analogy with the in vivo situation. Other in vitro systems which have incorporated continuous-flow dialysis are those of Davey et al. (1959) and Gray et al. (1962) who used relative flow rates of 2.45 and 6.66 ml/h/100 ml of culture respectively. The chosen F_d was a compromise between a high rate, for maximum removal of VFA, and lower rate to allow detection and measurement of VFA in the dialysate. In preliminary experiments a F_d of 60 ml/h was found to be satisfactory in terms of resulting TVFA concentrations, giving values of 10 - 15 m mol/l. This represents a relative flow rate of 14.63 ml/h/100 ml of culture.

Passage of O_2-free gas through the culture has no parallel in vivo. Rates of flow quoted for previous in vitro work are less than definitive eg. "slow stream", (Warner 1956); "gassed continuously", (El Shazly et al. 1960); "blown through", (Davey et al. 1960); "passage of ... through the vessel", (Gray et al. 1962). The maximum flow rate of gases (F_g) which could be achieved with both meters reading full scale, was 1200 ml/min. Since the mixture of gases used was 95% N_2 : 5% CO_2 the greatest F_g available was about half the above figure. The minimum practical F_g was that which ensured transport of effluent from the vessel and prevented fibrous aggregates forming in the effluent weir. This was

200 ml/min and it was decided that a suitable working F_g value was 200 ml/min.

c) Inoculum. At the start of each experimental period the culture vessel was inoculated with rumen liquor strained through two layers of muslin. The entire volume of the culture was made up of rumen liquor from 3 Cheviot wether sheep maintained on a constant diet of hay and concentrates. The rumen liquor was removed and transported by the methods described for sampling in vivo (page 91).

The culture vessel was gassed, before inoculation via the pH electrode port. The temperature control system was switched on as soon as working volume was reached. After insertion of the pH electrode, pH, antifoam and feed controls were switched on, and the recorder started.

d) Culture period. The criteria of steady state working were : 1) stable NH_3 concentration (chosen because analytical results were available within a few hours of sampling), 2) consistency of demand for pH adjustment and 3) stability of the redox potential. The times taken to reach steady state were similar to those of Slyter et al. (1964).

Volumes of liquid in the reservoirs and effluent reception vessel were recorded initially and daily, as well as during each sampling period. Substrate was stored in bulk under refrigeration, the feed reservoirs

being replenished daily.

e) Sampling. Samples of the contents of the culture vessel were withdrawn via the gassing port, after detaching the gas line. This eliminated any risk of culture discharge, owing to gas pressure, during sampling. A modified 10 ml glass syringe, fitted with a 7 mm diameter glass extension nozzle was used. The sample was drawn into the extension tube only. An appropriate volume was discharged into the sample receiving vessel, using the syringe calibrations as a volumetric guide.

Samples of dialysate were taken directly from the dialysate discharge into a tube cooled in ice. Acidification and precipitation of samples for VFA analysis was carried out promptly.

During each sampling period, samples were withdrawn according to a fixed schedule, detailed in Appendix 2.M. Methods of analysis are detailed elsewhere (Section 4).

DATA RECORDED

In addition to chemical and microbiological analysis, and the chart recorder traces, total deliveries of substrate (V_s), buffer (V_b), antifoam (V_a) and dialysis solution (V_d) were recorded during each experimental period. Whilst V_s, V_a and V_d were nominally the same for each experimental period, small differences did

arise and, together with the differences in V_b, resulted in variations in D. Since V_s and substrate DM were the same for each experiment, the DM content of the culture vessel varied only according to the value of D. Small adjustments to analytical results were therefore necessary to relate all values to substrate DM.
ie.

$$\text{Concentration estimated in culture} \times \frac{F}{V_s} \quad \ldots\ldots(7.1)$$

The value of F/V_s differed for each experimental period and is denoted F_{fac} in subsequent tables of results.

Similarly, in calculating TVFA (including dialysed VFA) simple summation of culture and dialysate concentration would have led to an erroneous value. VFA concentrations in culture and dialysate were in dynamic equilibrium and, since membrane effects are constant (Thomson 1973) the apparent concentration in dialysate could be multiplied by the ratio of culture and dialysate flow rate such that the volumes dialysed in unit time are compared.
ie.

$$C_d \times \frac{F_d}{F} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(7.2)$$

F_d/F is designated D_{fac} in subsequent tables of results. The value of TVFA was thus obtained by adding the result of calculation by equation (2) to the culture concentration (C_c). Subsequent multiplication by F_{fac} yield values suitable for comparison with those of other experiments.

STATISTICAL TREATMENT OF RESULTS

The steady-state kinetics of a Rumenstat in-vitro culture imply a stable and highly reproducible culture system varying only according to the nature of the substrate employed. Replicate runs with a single silage confirmed this and results for the material with which most replications were made (S 37; Appendix 3.C) show that the coefficients of variance (CV) between samples in single runs (ie within runs) compare with those of the mean values of replicate runs (ie between runs). For example :-

a) NH_3-N concentration; mean CV within runs : 7.89, CV between runs : 3.13.

b) TVFA concentration; mean CV within runs : 1.07, CV between runs : 2.94.

The main differences within any run were between the samples, and differences between replicates of any single sample were small in comparison. Differences between silages were therefore tested by applying the t-test to the steady-state sample means. Results presented are means of three samples at steady-state.

RESULTS.

Results presented here are for Rumenstat cultures of Group I, Group II and Group III silages. Details of treatments and analysis of these silages are given in Tables 11 - 13 (Section 5 pages 85 to 88).

GROUP I SILAGES.

1. Rumenstat Data.

Details of Rumenstat cultures with the Group I silages are given in Table 22. The abbreviations used in this table are detailed on page vi and on the insert card.

Feed and overall-flow rates (F_f and F) for these cultures were substantially different but correction factors for feed flow (F_{fac}) were similar, indicating that overall cultural characteristics were alike.

2. NH_3-N.

Analytical values of NH_3-N in the Rumenstat cultures are presented graphically in Figure 21. Table 23 gives mean steady-state concentrations.

NH_3-N concentrations with the control and formic acid/acetic acid silages were similar and close to the initial culture concentrations (Figure 21). A low concentration of NH_3-N was recorded with the formaldehyde/acetic acid treated material.

Table 22. Rumenstat data with Group I silages.

	silage		
	control	formic acid/ acetic acid	formaldehyde/ acetic acid
culture period (h)	130	119	146
V_f (ml)	1400	1576	2545
F_f (ml/h)	10.77	13.24	17.43
V_b (ml)	569	585	790
F_b (ml/h)	4.38	4.92	5.41
V_a (ml)	100	160	430
F_a (ml/h)	0.71	1.35	2.95
V_d (ml)	7510	7235	10205
F_d (ml/h)	57.77	60.80	69.90
V_t (ml)	2069	2321	3765
F (ml/h)	15.92	19.50	25.79
D	0.039	0.048	0.063
T_r (d^{-1})	0.958	1.41	1.51
T_t (h)	25.76	21.02	15.89
F_{fac}	1.478	1.473	1.479
D_{fac}	3.629	3.117	2.711

Table 23. Mean concentrations of NH_3-N (mg/l) with Group I silages at steady-state.

	Silage		
	control	formic acid/ acetic acid	formaldehyde/ acetic acid
measured concentration	189	179	81
X F_{fac}	300 a	259 a	120 b

a, b; means without the same letter differ significantly ($P < 0.01$)

3. VFA.

The measured concentration of TVFA are shown in Figure 22. Mean steady-state molar percentages are given in Table 24. All concentrations are inclusive of dialysed VFA.

The additive-treated silages resulted in lower TVFA than the control. The proportion of acetate in the TVFA was low in the case of the control silage (55.2) and high with the formaldehyde/acetic acid treatment (73.1). The control silage resulted in a high concentration of butyrate (17.7 %) whilst the formaldehyde/acetic acid treated material resulted in low propionate (11.00 %).

4. Differential Counts.

Counts made on starch, gelatin and cellulose media are shown graphically in Figures 23, 24 and 25. Mean steady-state values are given in Table 25.

Table 24. Mean molar percentages of VFA at steady-state; Group I silages.

	TVFA (XF_{fac})	C_2 C^2	C_3	iC_4	C_4	iC_5	C_5	C_6
control	300.7a	55.2	21.7	1.0	17.7	1.7	1.7	1.0
formic acid / acetic acid	152.4b	67.5	13.8	1.2	11.8	2.4	1.9	1.4
formaldehyde/ acetic acid	113.72c	73.1	11.0	1.0	8.8	1.2	1.3	3.5

a,b,c, Means without the same letter differ significantly ($P < 0.01$)

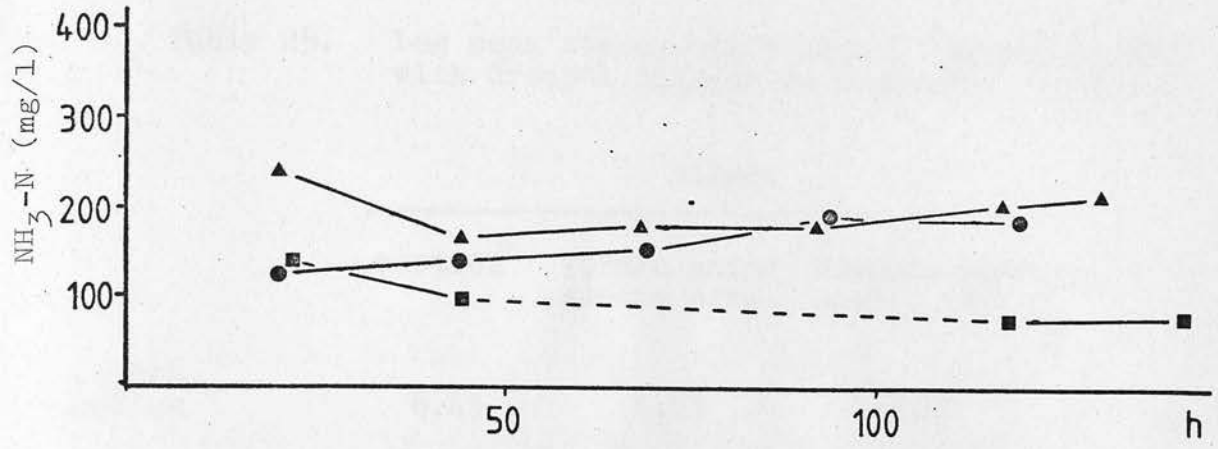


Fig. 21 Group I silages, analytical values of NH_3-N (mg/l) in Rumenstat culture.

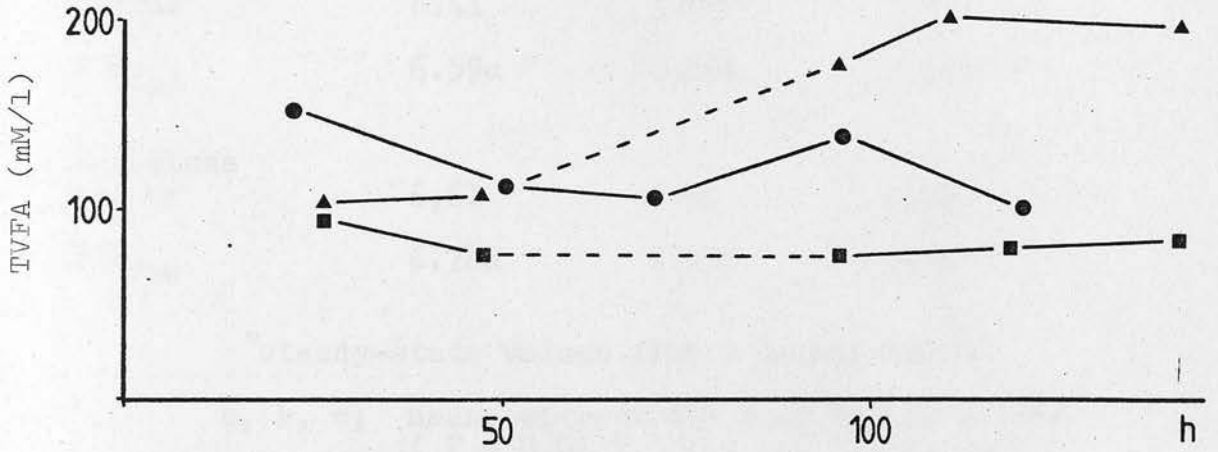


Fig. 22 Group 1 silages, analytical values of TVFA (mM/l) in Rumenstat culture.

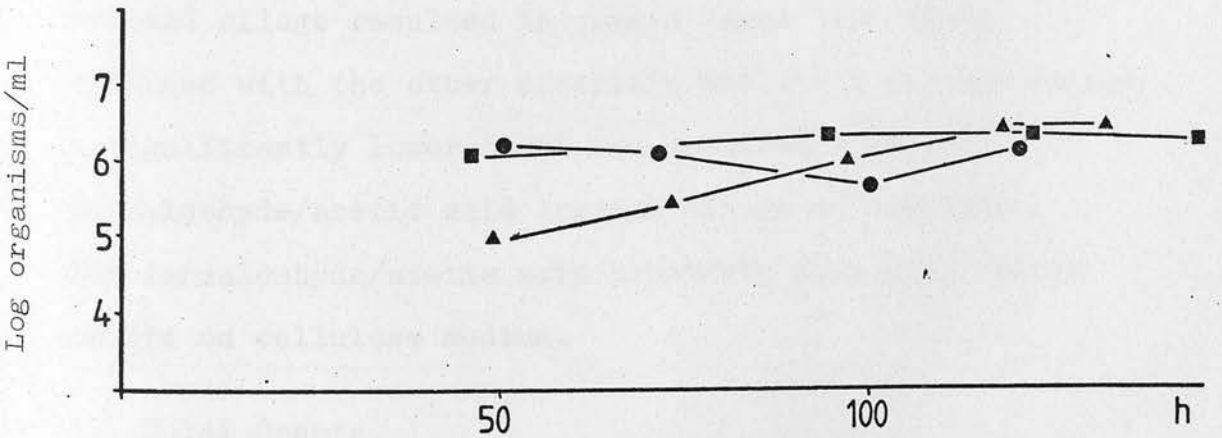


Fig. 23 Group 1 silages, log viable counts on starch medium in Rumenstat culture.

▲control, ●formic acid/acetic acid, ■formaldehyde/acetic acid

Table 25. Log mean steady-state counts (organsims/ml) with Group 1 silages in Rumenstat culture.

	silage		
	control$^{\nabla}$	formic acid/ acetic acid	formaldehyde/ acetic acid
starch medium	6.45	6.03	6.33
X F_{fac}	6.62a	6.20b	6.50a
gelatin medium	6.41	5.99	5.35
X F_{fac}	6.59a	6.16a	5.52b
cellulose medium	6,61	6.03	5.44
X F_{fac}	6.78a	6.17b	5.61c

$^{\nabla}$steady-state values from 2 measurements

a, b, c; means without the same letter differ ($P < 0.01$)

On starch medium the formic acid/acetic acid treated silage resulted in counts lower than those obtained with the other materials whilst on gelatin medium a significantly lower count was obtained with the formaldehyde/acetic acid treated silage as substrate. The formaldehyde/acetic acid treatment also gave lowest counts on cellulose medium.

5. Total Counts.

Bacterial and protozoal total counts are shown

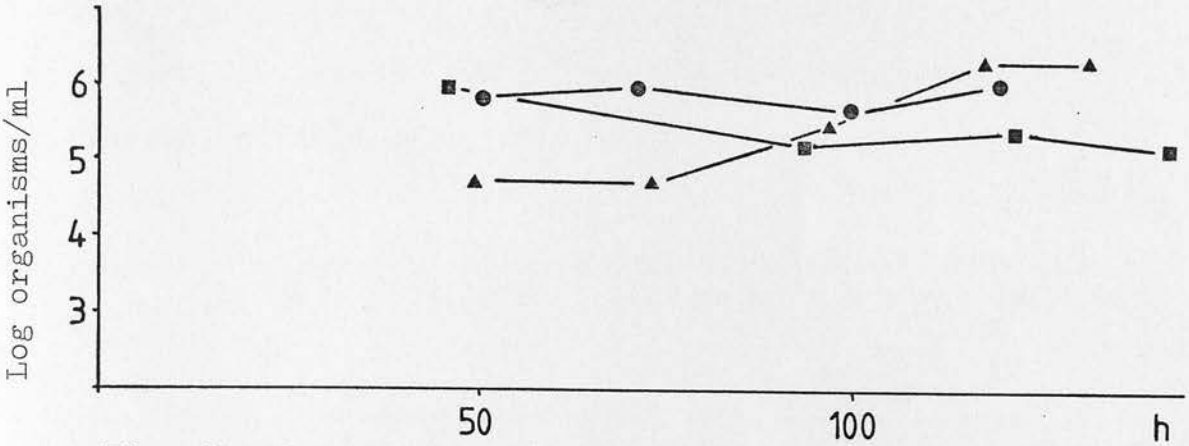


Fig. 24 Group 1 silages, log viable counts on gelatin medium in Rumenstat culture.

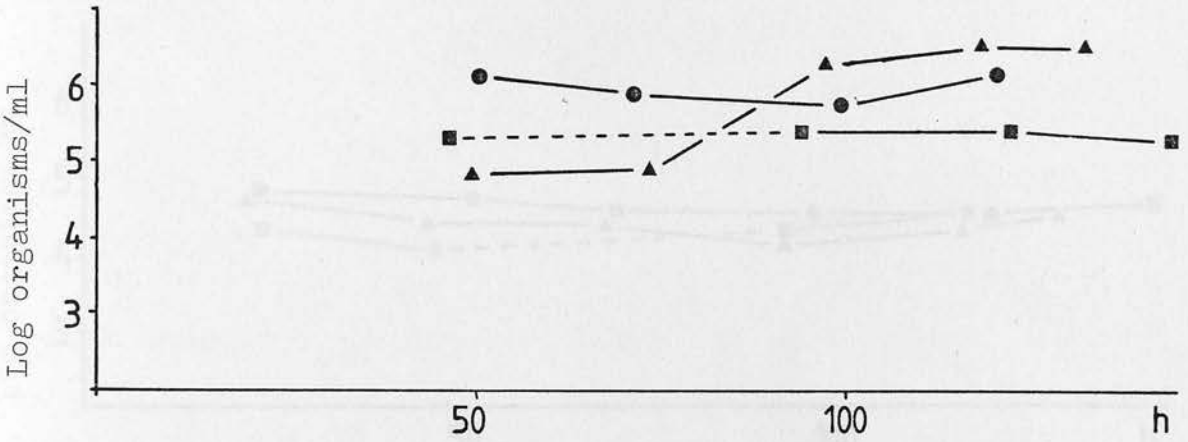


Fig. 25 Group 1 silages, log viable counts on cellulose medium in Rumenstat culture.

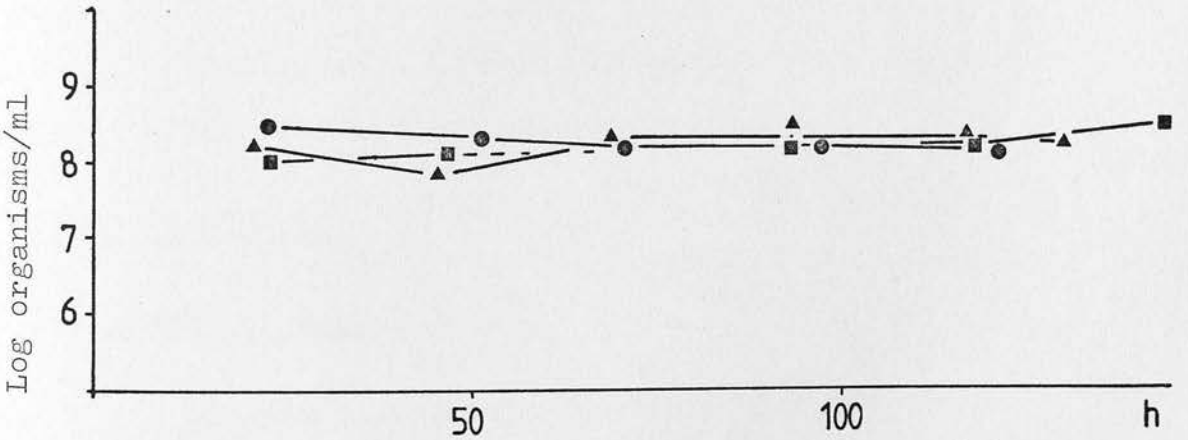


Fig. 26 Group 1 silages, log total bacterial counts in Rumenstat culture.

▲control, ●formic acid/acetic acid, ■formaldehyde/acetic acid

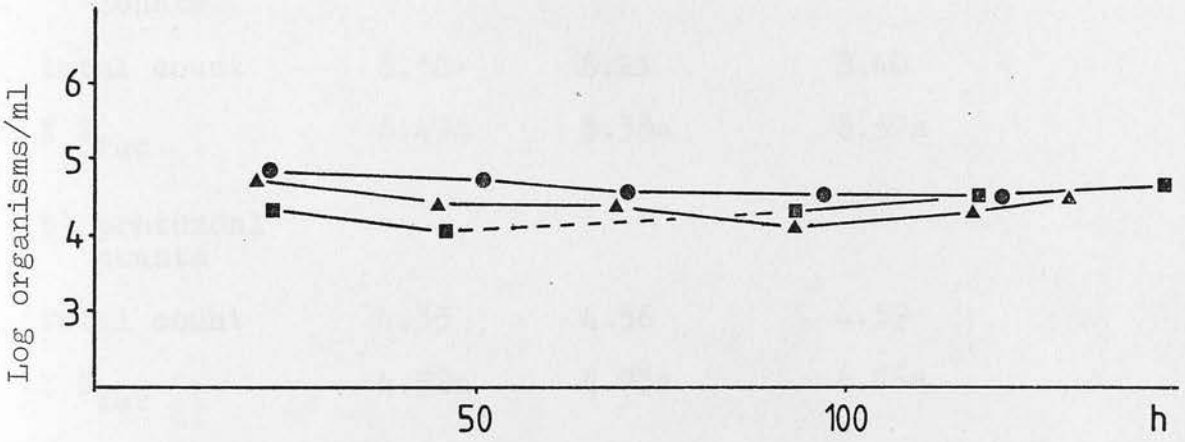


Fig. 27 Group 1 silages, log total protozoa counts in Rumenstat culture.

▲ control, ● formic acid/acetic acid, ■ formaldehyde/acetic acid

in Figures 26 and 27. Mean steady-state counts are presented in Table 26.

Table 26. Log mean steady-state total counts (organisms/ml) with Group 1 silages in Rumenstat culture.

	silages		
	control	formic acid/ acetic acid	formaldehyde/ acetic acid
a) bacterial counts			
total count	8.32	8.21	8.40
X F_{fac}	8.49a	8.38a	8.57a
b) protozoal counts			
total count	4.35	4.56	4.59
X F_{fac}	4.52a	4.73a	4.76a

a; means did not differ significantly ($P < 0.01$)

Total counts of bacteria and protozoa did not show differences between silage treatments.

GROUP II SILAGES.

1. Rumenstat Data.

Details of Rumenstat cultures with the Group II silages are given in Table 27.

Table 27. Rumenstat data with Group II silages.

	Silage			
	control	wilted	fresh/ formic acid	wilted/ formic acid
culture period (h)	114	118	99	118
V_f (ml)	1160	1220	1145	1220
F_f (ml/h)	10.18	10.34	11.57	10.34
V_b (ml)	380	330	280	440
F_b (ml/h)	3.35	2.80	2.83	3.73
V_a (ml)	70	80	125	100
F_a (ml/h)	0.62	0.68	1.26	0.85
V_d (ml)	7230	7086	7020	8870
F_d (ml/h)	63.42	60.05	70.92	75.17
V_t (ml)	1610	1630	1550	1760
F (ml/h)	14.12	13.81	15.66	14.92
D	0.034	0.034	0.038	0.036
T_r (d^{-1})	0.827	0.809	0.916	0.873
T_t (h)	29.03	29.68	26.19	27.49
F_{fac}	1.389	1.336	1.354	1.443
D_{fac}	4.491	4.347	4.529	5.039

Feed flow rates were similar with this group of silages but substantial demand for pH correction in the case of the wilted/formic acid material, resulted in a high value of F_{fac} (1.443) compared with the other cultures in this group.

2. NH_3-N.

Analytical values of NH_3-N obtained with the Group II silages are presented graphically in Figure 28. Table 28 gives mean steady-state concentrations.

Table 28. Mean concentrations of NH_3-N (mg/l) with Group II silages at steady-state.

	silage			
	control	wilted	fresh/ formic acid	wilted/ formic acid
measured concentration	217	259	261	211
X F_{fac}	301 a	377 b	353 b	305 a

a, b; means without the same letter differ significantly ($P < 0.01$)

NH_3-N concentrations with the wilted and formic acid-treated fresh material were higher than those with the control and formic acid-treated wilted materials.

3. VFA

The measured concentrations of TVFA obtained

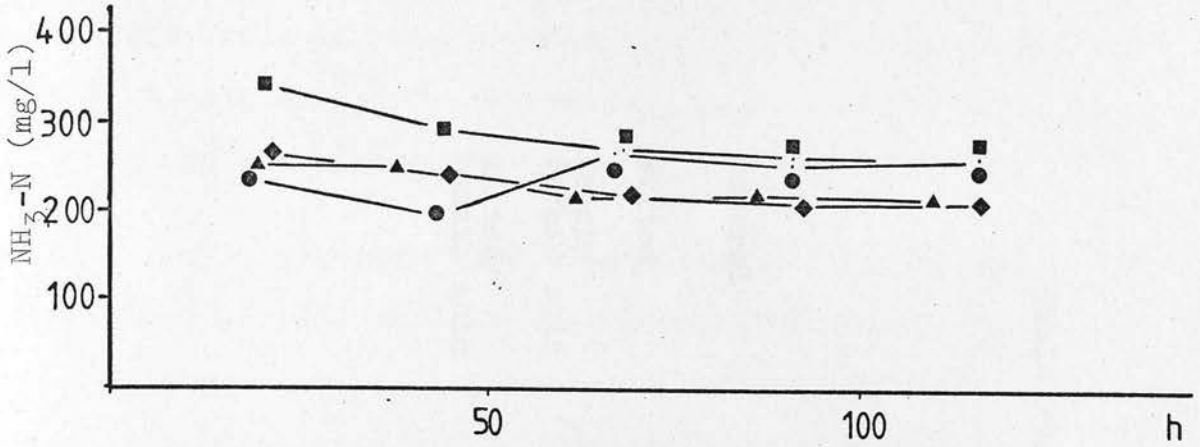


Fig. 28 Group 2 silages, analytical values of NH_3-N (mg/l) in Rumenstat culture.

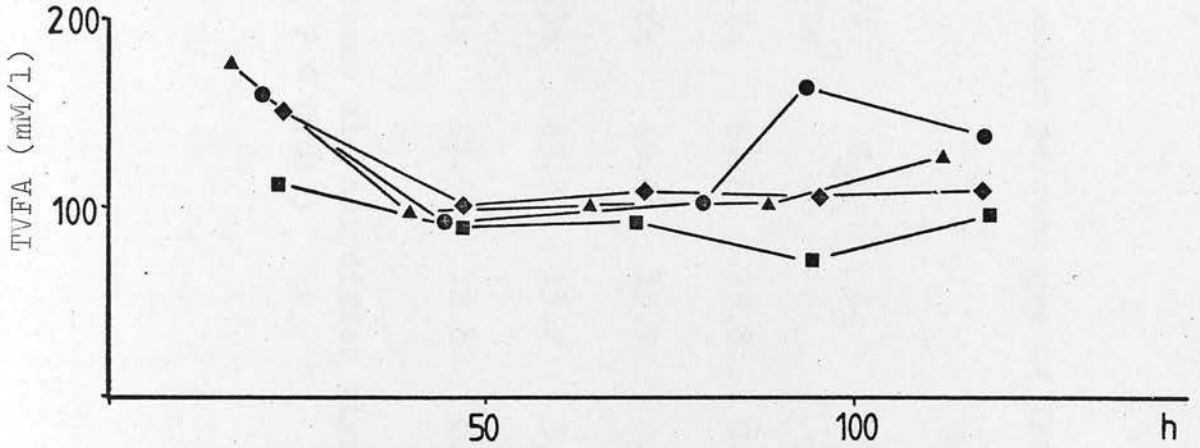


Fig. 29 Group 2 silages, analytical values of TVFA (mM/l) in Rumenstat culture.

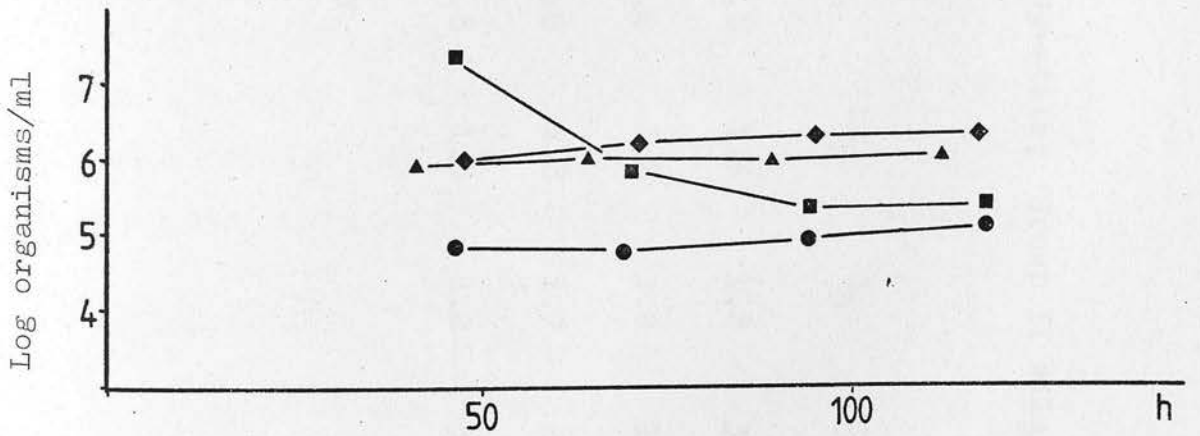


Fig. 30 Group 2 silages, log viable counts on starch medium in Rumenstat culture

▲fresh, ●wilted, ■fresh/formic acid, ◆wilted/formic acid

Table 29. Mean molar percentages of VFA at steady-state; Group II silages.

	TVFA ($X F_{fac}$)	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
control	160.7a	61.8	19.8	0.9	10.8	1.6	1.2	3.9
wilted	183.8a	69.6	15.7	0.8	9.4	1.8	1.6	1.1
fresh/ formic acid	118.23a	65.5	13.2	3.2	9.6	4.8	1.7	2.1
wilted/ formic acid	154.71a	69.9	12.8	0.7	10.8	1.3	1.2	3.3

a; means did not differ significantly
($P < 0.01$)

with the Group II silages are shown in Figure 29. Mean steady-state molar percentages are given in Table 29. All concentrations are inclusive of dialysed VFA.

TVFA concentrations showed a fall from initial values of 160-180 mM/l to 100-130 mM/l (uncorrected) at steady-state. The differences in TVFA concentrations were not significant. High proportions of acetic acid were measured with the wilted silages (69.6 and 69.9) and all but the untreated wilted silage resulted in a large proportion of C_6 acid.

4. Differential Counts.

Counts made on starch, gelatin and cellulose media are shown graphically in Figures 30, 31 and 32. Mean steady-state values are given in Table 30.

Counts on starch medium with the control and wilted silages were lower than those obtained with the treated silages. The initial high counts on the formic acid-treated fresh material (Fig. 30) declines to values lower than those obtained with the formic acid-treated wilted material. On gelatin medium the treated materials gave counts significantly higher than the untreated.

5. Total Counts.

Bacterial and protozoal counts are shown in Figures 33 and 34. Mean steady-state counts are presented in Table 31.

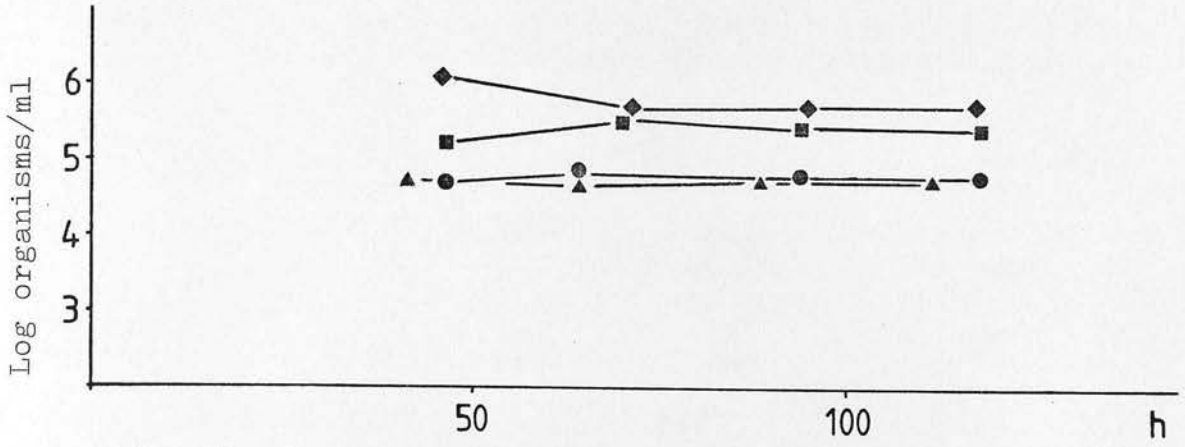


Fig. 31 Group 2 silages, log viable counts on gelatin medium in Rumenstat culture.

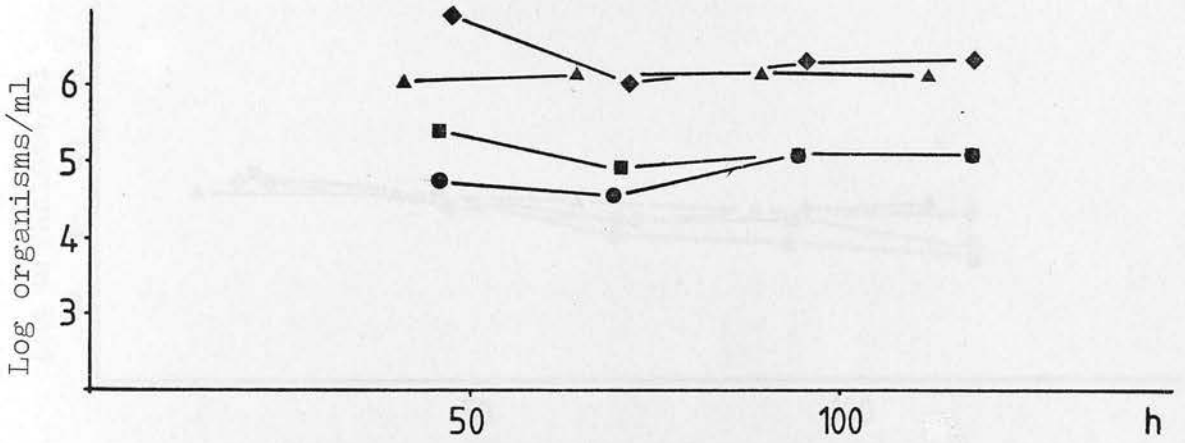


Fig. 32 Group 2 silages, log viable counts on cellulose medium in Rumenstat culture.

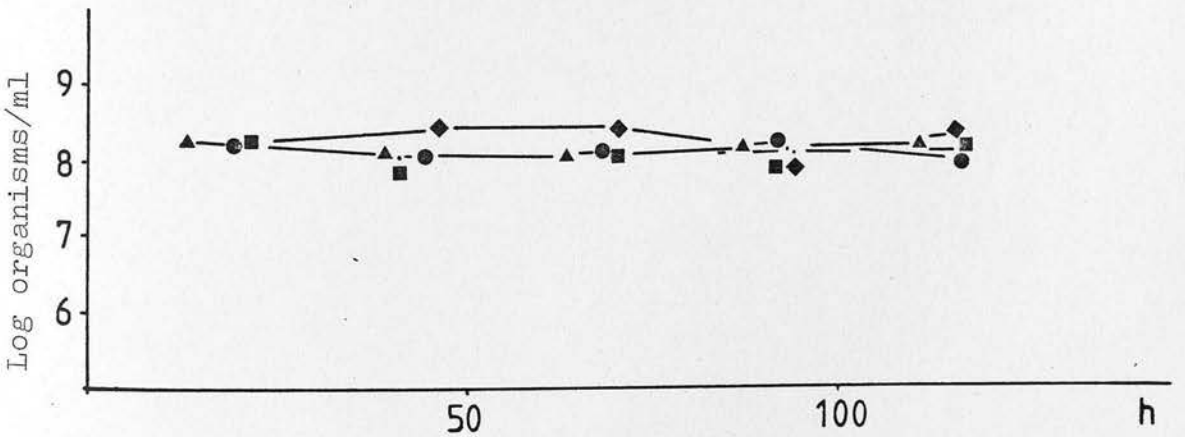


Fig. 33 Group 2 silages, log total bacterial counts in Rumenstat culture.

▲ fresh, ● wilted, ■ fresh/formic acid, ◆ wilted/formic acid

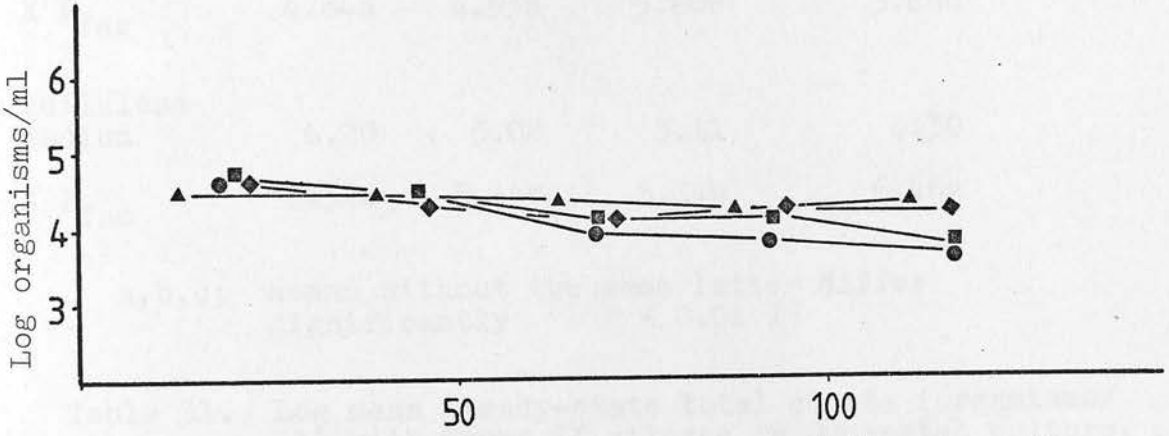


Fig. 34 Group 2 silages, log total protozoal counts in Rumenstat culture.

▲ fresh, ● wilted, ■ fresh/formic acid, ◆ wilted/formic acid

Table 30. Log mean steady-state counts (organisms/ml) with Group II silages in Rumenstat culture.

	silage			
	control	wilted	fresh/ formic acid	wilted/ formic acid
starch medium	5.04	4.99	5.62	5.62
X F_{fac}	5.19a	5.08a	5.75b	6.47c
gelatin medium	4.70	4.81	5.47	5.72
X F_{fac}	4.84a	4.93a	5.60b	5.88c
cellulose medium	6.20	5.02	5.11	6.30
X F_{fac}	6.34a	5.14b	5.24b	6.46a

a,b,c; means without the same letter differ significantly ($P < 0.01$)

Table 31. Log mean steady-state total counts (orgamisms/ ml) with Group II silages in Rumenstat culture.

	silage			
	control	wilted	fresh/ formic acid	wilted/ formic acid
a) bacteria counts				
total count	8.17	8.14	8.11	8.31
X F_{fac}	8.31a	8.43a	8.25a	8.47a
b) protozoa counts				
total count	4.35	3.85	4.04	4.70
X F_{fac}	4.49a	3.98b	4.17a	4.35a

a, b; means without the same letter differ significantly ($P < 0.01$)

GROUP III SILAGES.

1. Rumenstat Data.

Details of Rumenstat cultures with Group III silages are given in Table 32.

Table 32. Rumenstat data with Group III silages.

	silage			
	control	f/dehyde / formic acid	f/dehyde/ H_2SO_4	f/dehyde
culture period (h)	283	142	125	142
V_f (ml)	3170	1350	1285	1260
F_f (ml/h)	11.20	9.51	10.28	8.80
V_b (ml)	775	610	295	415
F_b (ml/h)	2.74	4.30	2.36	2.92
V_a (ml)	350	170	240	155
F_a (ml/h)	1.24	1.20	1.92	1.09
V_d (ml)	15660	10380	8050	8290
F_d (ml/h)	55.34	73.1	64.4	58.38
V_t (ml)	4295	2130	1820	1830
F (ml/h)	15.18	15.00	14.56	12.89
D (h)	0.037	0.037	0.036	0.031
T_r (d^{-1})	0.887	0.878	0.852	0.754
T_t (h)	27.05	27.33	28.16	31.82
F_{fac}	1.355	1.578	1.416	1.452
D_{fac}	3.660	4.873	4.423	4.530

(f/dehyde = formaldehyde)

Turnover times with the Group III silages were similar but different proportions of buffer contributing to overall culture flow (F) resulted in a range of values for F_{fac}.

2. $\underline{NH_3\text{-}N}$.

Analytical values of NH_3-N obtained with the Group III silages are presented graphically in Figure 35. Table 33 gives mean steady-state concentrations.

Table 33. Mean concentrations of NH_3-N (mg/l) with Group III silages at steady-state.

	silage			
	control	f/dehyde/ formic acid	f/dehyde H_2SO_4	f/dehyde
measured concentration	239	129	299	233
X F_{fac}	323 a	203 b	424 c	339 a

a, b, c; means without the same letter differ significantly ($P < 0.01$)

(f/dehyde = formaldehyde)

A low concentration of NH_3-N was measured with the formaldehyde/formic acid treated material whilst the highest NH_3-N concentration with this group arose with the formaldehyde/H_2SO_4 silage. The control and formaldehyde-only treated materials did not result in significantly different concentrations of NH_3-N.

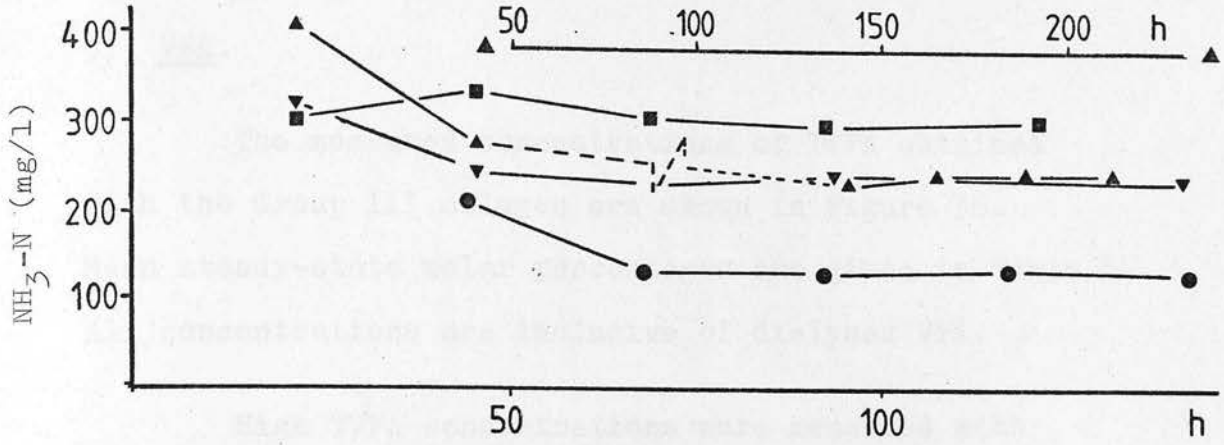


Fig. 35 Group 3 silages, analytical values of NH_3-N (mg/l) in Rumenstat culture.

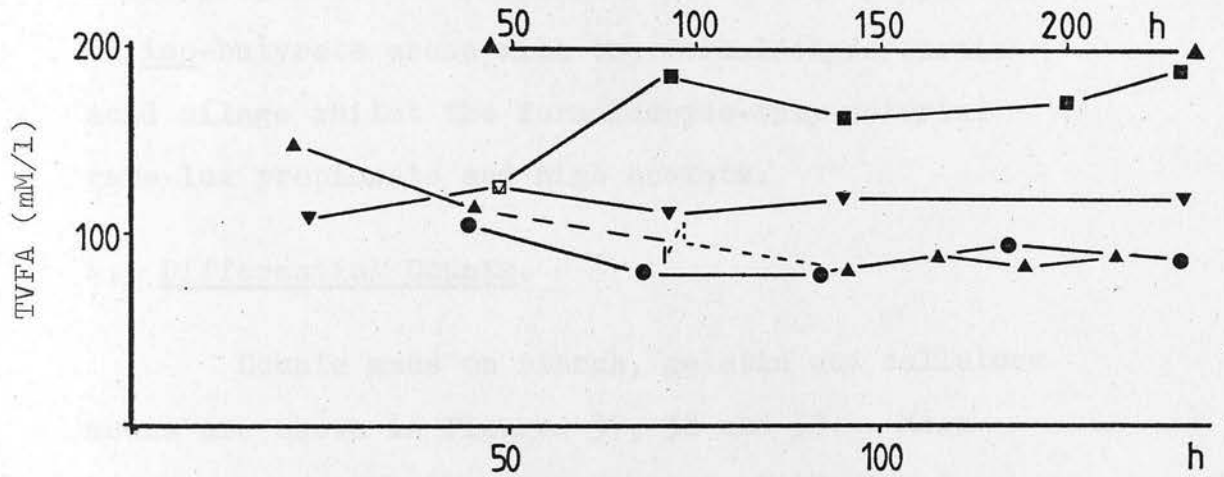


Fig. 36 Group 3 silages, analytical values of TVFA (mM/l) in Rumenstat culture.

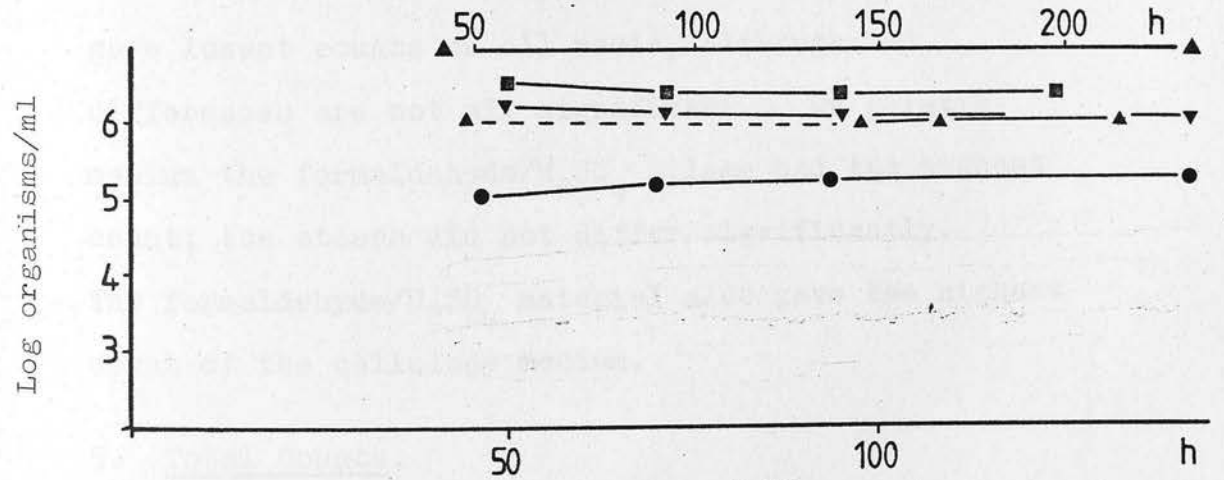


Fig. 37 Group 3 silages, log viable counts on starch medium in Rumenstat culture.

▲ control, ● formaldehyde/formic acid,
■ formaldehyde/H_2SO_4, ▼ formaldehyde

3. VFA.

The measured concentrations of TVFA obtained with the Group III silages are shown in Figure 36. Mean steady-state molar percentages are given in Table 34. All concentrations are inclusive of dialysed VFA.

High TVFA concentrations were measured with the formaldehyde/H_2SO_4 and formaldehyde-only silages. A low proportion of propionate and a high proportion of iso-butyrate arose with the formaldehyde/formic acid silage whilst the formaldehyde-only material gave low propionate and high acetate.

4. Differential Counts.

Counts made on starch, gelatin and cellulose media are shown in Figures 37, 38 and 39. Mean steady-state values are given in Table 35.

The formaldehyde/formic acid treated silage gave lowest counts on all media, although the differences are not all significant. On gelatin medium the formaldehyde/H_2SO_4 silage had the highest count; the others did not differ significantly. The formaldehyde/H_2SO_4 material also gave the highest count of the cellulose medium.

5. Total Counts.

Bacterial and protozoal total counts are shown in Figures 40 and 41. Mean steady-state counts are presented in Table 36.

Table 34. Mean molar percentages of VFA at steady-state; Group III silages.

	TVFA ($X F_{fac}$)	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
control	119.6a	65.9	14.8	1.5	12.8	2.5	1.5	1.0
formaldehyde/ formic acid	148.7a	69.2	10.6	5.5	9.8	2.1	1.7	1.0
formaldehyde/ H_2SO_4	247.7b	69.4	15.8	1.1	9.3	2.5	1.4	0.7
formaldehyde	169.9c	71.0	12.0	0.9	10.6	2.6	1.8	1.2

a, b, c; means without the same letter differ significantly ($P < 0.01$)

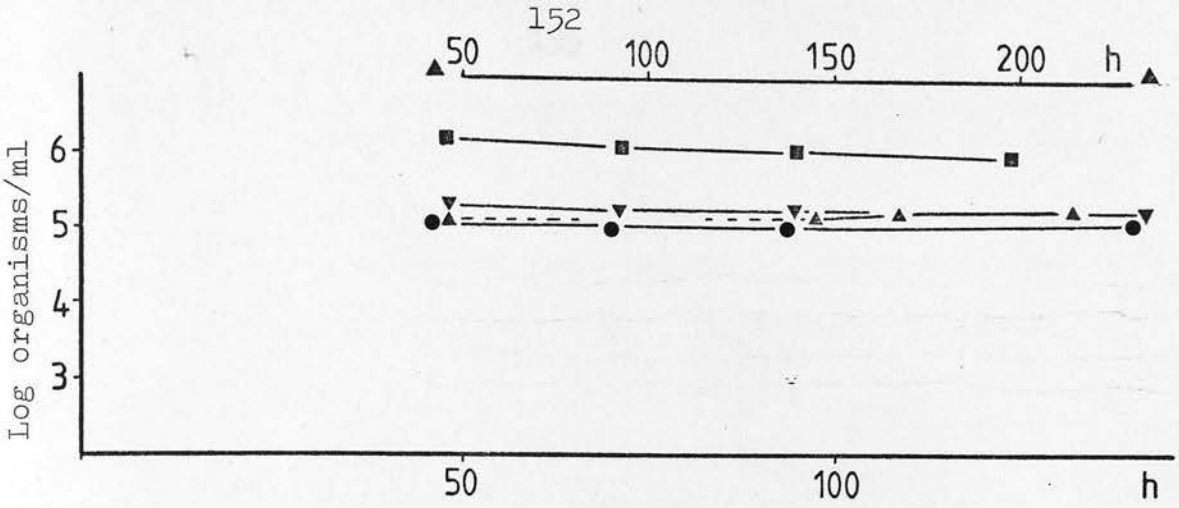


Fig. 38 Group 3 silages, viable counts on gelatin medium in Rumenstat culture.

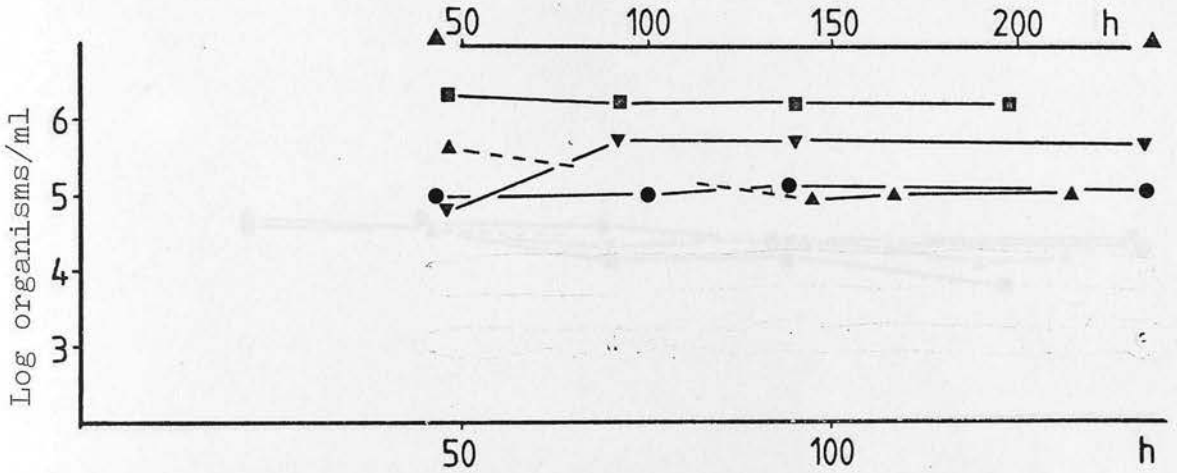


Fig. 39 Group 3 silages, viable counts on cellulose medium in Rumenstat culture.

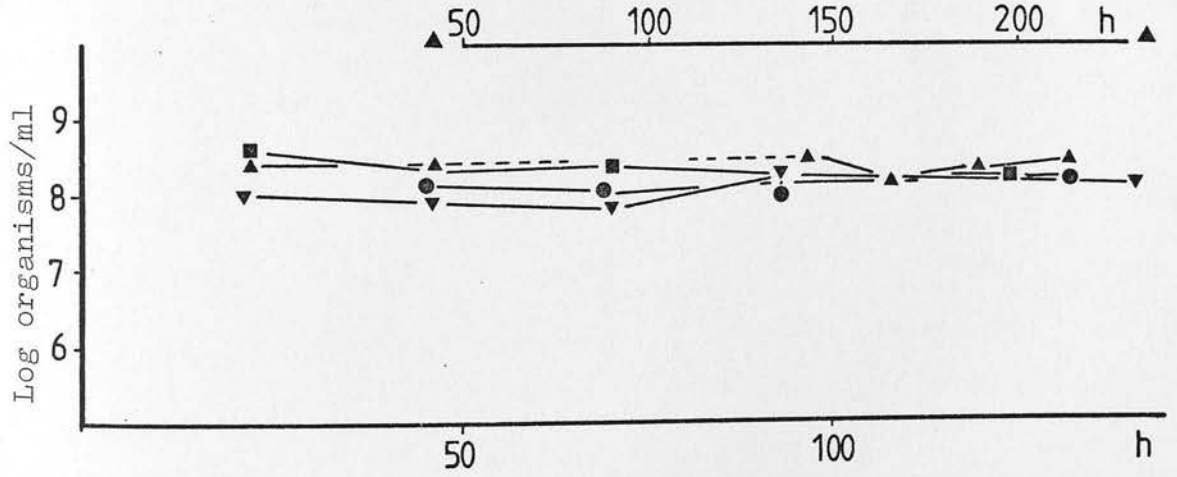


Fig. 40 Group 3 silages, total bacterial counts in Rumenstat culture.

▲ control, ● formaldehyde/formic acid,
■ formaldehyde/H_2SO_4, ▼ formaldehyde

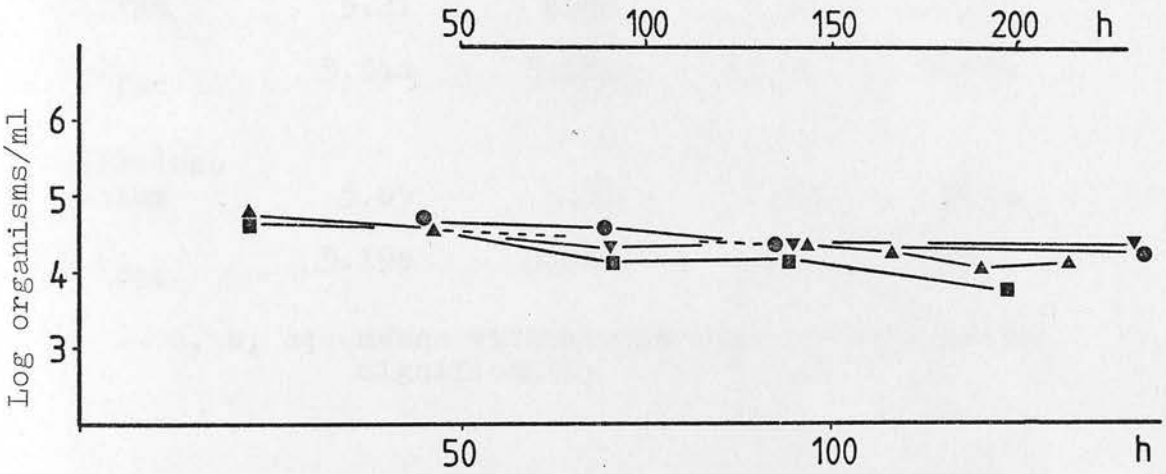


Fig. 41 Group 3 silages, total protozoal counts in Rumenstat culture.

▲control, ●formaldehyde/formic acid,
■formaldehyde/H_2SO_4, ▼formaldehyde

Table 35. Log mean steady-state counts (organisms/ml) with Group III silages in Rumenstat culture.

	silage			
	control	f/dehyde/ formic acid	f/dehyde/ H_2SO_4	f/dehyde
starch medium	6.03	5.22	6.45	6.12
X F_{fac}	6.16a	5.42b	6.61c	6.28a
gelatin medium	5.21	6.08	6.02	5.22
X F_{fac}	5.34a	5.28a	6.17b	5.38a
cellulose medium	5.05	5.08	6.23	5.73
X F_{fac}	5.19a	5.28a	6.38b	5.89c

a, b, c; means without the same letter differ significantly ($P < 0.01$)

Table 36. Log mean steady-state total counts (organisms/ml) with Group III silages in Rumenstat culture.

	silage			
	control	f/dehyde/ formic acid	f/dehyde/ H_2SO_4	f/dehyde
a) bacteria counts				
total count	8.35	8.15	8.30	8.13
X F_{fac}	8.48a	8.34a	8.45a	8.29a
b) protozoa				
total counts	4.17	4.38	4.09	4.34
X F_{fac}	4.30a	4.59a	4.24a	4.50a

a; means did not differ significantly

DISCUSSION

There was a high positive correlation between in vitro NH_3-N concentration and microbial counts on the gelatin medium for the Group I and Group III silages ($r = 0.98$ and 0.80 respectively). Lowest values were generally associated with formaldehyde-treated silages, but the formaldehyde/H_2SO_4 treatment in Group III showed a high in vitro NH_3-N concentration (424 mg/l) and high counts on the gelatin medium (6.17 (log_{10})/ml). This accords with the silage composition which had a high nitrogen content (32.1 g/kg DM), low WSC (64 g/kg DM) and a high proportion of NPN (0.51 of TN). It would appear that fermentation of this material in the silo had been inhibited less than the others in this group and protein binding by formaldehyde, had been limited. This is in keeping with the lower rate of application of formaldehyde used in ensiling this material. The in vivo data (Table 17 and page 95) confirm the high NH_3-N and gelatin counts for the formaldehyde/H_2SO_4 treated material and the high TVFA measured in vitro (247.7 mM/l) suggests a high level of microbial metabolism which would only be possible if the microflora were not nitrogen limited.

It is a characteristic of continuous culture that the size of the microbial population (the so-called "biomass") is determined chiefly by the

concentration of the growth-limiting substrate in the culture medium (Appendix 1.A cf. p. 62). In a mixed continuous culture the types and numbers of constituent species will be determined largely by the nature of the growth-limiting substrate.

On a silage diet the rumen microflora obtain readily available energy from residual WSC and the products of in-silo fermentation. The energy value of the latter to the rumen microorganisms is problematical. Although the gross energy of these fermentation products may be high, eg. lactic acid and ethanol have energy values of 15.2 and 29.8 MJ/kg respectively, this may not reflect their usefulness to the microflora. The potential of in-silo fermentation products of WSC, as microbial energy sources in the rumen, calculated for a typical lactate silage, is summarised below :

For 1g of silage DM [a] :

silage component	wt	wt of original hexose	mM component	ATP yield/M	mM ATP	
lactate	120	120	1.33	1.29	1.72	b
ethanol	10	19.5	0.22	≈ 1.0	0.22	c
mannitol	60	59.6	0.33	4.58	1.51	d
others	20	30.0	0.23	≈ 1.67	0.38	c
		----			----	
total WSC fermented in silo =		229.1	ruminal ATP yield of products =		3.83	

---

a. Silage composition based on McDonald and Whittenbury (1973).

b. Calculation based on Baldwin et al.(1969) and assuming no energy expenditure in lactate → pyruvate.

c. Estimated from Baldwin et al. (1969)

d. Calculation based on Baldwin et al. (1969) and assuming no energy expenditure in mannitol → fructose.

The ruminal energy potential of the original WSC, before in silo formentation, is shown below :

Total consumed hexose for silage products above (mg)	=	229.1
ATP yield/m	=	4.58 a
mM hexose	=	1.27
ruminal ATP yield	=	5.82

The loss of potential energy resulting from fermentation in silo is thus of the order of $\frac{5.82 - 3.83}{5.82} = 0.342$. A further potential limitation of the silage fermentation products as energy sources may be the extent to which they are dissimilated in the rumen and the speed with which this takes place. Lactic acid has been shown to be rapidly decomposed (Thomson 1973) but the degradability of more esoteric constituents such as iso-pentanol, mannitol and various esters may be less extensive and slow. Under these circumstances the importance of WSC as a readily-available energy source will be accentuated and the level present in the diet may be critical in deciding the nature, numbers and activities of the rumen microorganisms.

Where WSC is available, organisms adapted to its utilisation will be encouraged. Where WSC is limited, cellulose and hemicelluloses, which are

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a : From Baldwin et al. (1969) assuming the greatest theoretical ATP yield, based on microbial yield data.

usually present in excess, will assume much greater importance and organisms capable of cellulolysis will establish dominance. The value of cellulose as an energy source is decided by the rate of cellulolysis, which is dependent upon a supply of available nitrogen. NH_3-N concentration is a good indicator of the nitrogen status of the culture, a low value implying limitation.

Where there is adequate WSC but N is limiting, organisms utilising soluble carbohydrates will tend to dominate a nutrient-limited continuous culture, and by their demands on the available nitrogen, bring about a nitrogen deficit for cellulolytic organisms. TVFA production will thus tend to be reduced by poor exploitation of the major substrate. At the other extreme, if WSC is restricted and nitrogen not limiting, the cellulolytic organisms will dominate the culture and, since their metabolism will not be restricted by nitrogen shortage, TVFA concentrations will be high.

Consideration of the figures given in the table overleaf leads to a classification of the experimental silages into three major types. At one extreme are those with high WSC but in which nitrogen was limiting, typified by silages 3 and 9. Micro-organisms capable of utilising the readily available WSC would have dominated the fermentation and monopolised the nitrogen resources of the culture.

Silage WSC and in vitro NH_3-N and TVFA figures for Group I, II and III silages.

			silage	in vitro	
group	number	treatment	WSC (mg/kg DM)	NH_3-N (mg/l)	TVFA (mM/l)
I	1	control	38.0	300	300
	2	formic/acetic acid	96.4	259	152
	3	formaldehyde/ acetic acid	144.5	120	113
II	4	control	7.1	301	160.7
	5	wilted	54.9	347	183.8
	6	fresh/formic acid	71.0	353	118.2
	7	wilted/formic acid	193	305	154.7
III	8	control	41.0	323	119.6
	9	formaldehyde/ formic acid	133	203	148.7
	10	formaldehyde/ H_2SO_4	64.0	424	247.7
	11	formaldehyde	151	339	169.9

The cellulolytic organisms would have been thus restricted and placed at a competitive disadvantage. The low TVFA measured with silage 3 in particular was probably the result of restricted cellulolysis.

The other extreme is the situation where nitrogen is not limiting and silage WSC concentration is low. Organisms capable of using this WSC only, would be placed at a competitive disadvantage, would not monopolise nitrogen and thus the cellulolytic organisms would be able to dominate the culture. Silages 1, 4, 8 and 10 were of this kind and the high TVFA measured in vitro with silages 1 and 10 may be explained on this basis. The very low WSC of silage 4 (7.1 g/kg DM) may explain the moderate TVFA value recorded but the low TVFA with silage 8 is anomalous and cannot be explained in the terms described above.

The other silages in these experiments belong to a third category with neither nitrogen nor readily available energy in the form of WSC, limiting. With these silages the microbial population in vitro would not be markedly biased towards either of the extremes postulated above. TVFA values were generally close to the mean of approximately 170 mM/l.

It is important to appreciate, when seeking to assess the relevance of information obtained using a steady-state in vitro system to the practical situation,

that the microbial population in vitro may be "switched" towards certain extremes by characteristics of the substrate which impose ecological stress on selected members of that population. Such circumstances would be unlikely to occur in the cyclical in vivo rumen situation except under extreme dietary conditions. Acidosis, for example, results from the ingestion of large quantities of grain by ruminants not accustomed to a grain diet. In acidosis, the organism S.bovis dominates the microflora, reducing the pH by the production of lactic acid. Likewise, under conditions of energy limitation, high levels of NH_3-N can arise and bring about ammonia toxicity (Juhasz 1962) particularly when ruminal pH is high.

The high counts on cellulose medium with formaldehyde-treated silages, which arose in vivo (Tables 15 and 17) did not occur in vitro, but there was a high correlation between counts on cellulose medium and TVFA concentration in vitro (r - Group I= 0.955, $p < 0.1$; r Group III = 0.953, $p < 0.05$). Since the cellulolytic species are major producers of acetate a relationship between their numbers and the proportion of acetate in the VFA might have been expected. No such relationship was found for any of the silage groups. It is known that silage diets tend to lead to a high proportion of acetate (Balch and Rowland 1957, Tilley et al. 1960 and Bath and Rook 1965) and that dietary

WSC content is negatively correlated with proportion of acetate (Anderson and Jackson 1971). The in vitro experiments reported here produced a contrary result, a positive correlation between silage WSC and proportion of acetate being established (p, all silages, $r < 0.05$). The relationship between silage WSC and in vitro TVFA already discussed (page 158), although non-significant, was consistently negative, indicating that the most extensive fermentation occurred with low WSC, non additive-treated silages and that, in general, such silages resulted in low proportions of acetate. In this context the positive relationship between counts of cellulolytic organisms and TVFA, whilst consistent with the previous observations concerning energy and nitrogen-availability, is apparently contrary to the measured proportions of acetate. It is possible that the substrate-limited conditions of a continuous culture system result in a substantial commensal population of soluble sugar utilising organisms supported by the cellulose digesters and contributing to the fermentation products. This effect would be enhanced by the small particle size of the fibre component in these cultures which, together with the continuous stirring of the culture, would lead to rapid dispe sal of the products of extracellular cellulase activity. The findings of Issacson, Hinds, Bryant and Owens (1975) that proportions of fermentation

products in continuous in vitro rumen culture are influenced by turnover time, suggest that at low turnover rates, proportions are dissimilar to those in vivo. The turnover rate of the cultures discussed here were low in the terms of Issacson et al. and this may account for the unexpected proportions of acetate. Further detailed work on the effects of turnover rate would be necessary to clarify this matter and this appears to be an area meriting further investigation.

The in vitro cultures were clearly influenced by silage composition and it is apparent that the effects of additive treatment were mediated through the changes they brought about in the nature of the silage and not by any direct microbiological effects of their residues.

8. IN VITRO INFUSION EXPERIMENTS.

INTRODUCTION

Results of in vitro experiments showed significant effects of chemical composition of silages upon the biochemistry and microbiology of in vitro cultures. These effects were sufficiently large to confuse or even mask any effects which residual amounts may have had. In order to examine the effects of additives per se , a series of experiments were conducted in which different additives were infused directly into cultures, with the same additive-free silage as substrate.

Ruminal concentrations of additives arising from the ingestion of additive-treated silages are likely to be low. Formic acid applied at the maximum commercial rate of 5 l/tonne, assuming no losses during ensilage (Henderson and McDonald 1971) followed by the dilution of 5 kg of silage in 25 l of rumen fluid, would result in a ruminal concentration of 22 mM/l. This calculated value represents the highest likely to arise; actual concentrations would thus be a small proportion of the 150 - 200 mM/l TVFA typically found in the rumen.

The steady-state in vitro rumen, with its accurate monitoring of flow rate, is particularly suitable for this kind of study. Moreover, concentrations of silage additive which may be

hazardous to rumen function and/or the host if administered in vivo, can be used in vitro without risk.

EXPERIMENTAL

The experiments were designed to expose a steady-state microflora to an additive concentration corresponding to that encountered in vivo when a treated silage is consumed, then gradually increasing the concentration so that specific effects could be detected.

Before commencement of additive infusion, the cultures were allowed three days for the establishment of steady-state on the basic silage "diet". After one day at steady-state, additives were infused by means of a calibrated pump. Incorporation of antifoam compound (polyethylene glycol; 100μ g/l) directly into the substrate allowed the pump, which otherwise delivered antifoam, to be used for infusion (Appendix 2.H).

Infusion Regime.

Infusion into a continuous - flow system requires careful planning and control, if flow-rates and concentrations are to be adequately regulated.

A pump delivering an additive solution of concentration C_{add} at rate F_{add} to a vessel of fixed volume V, will give rise to an increase in the additive concentration in the vessel (C_v) which may be

calculated as follows :-

$$\frac{d\,C_v}{dt} = \frac{F_{add} \times C_{add}}{V} \quad \ldots\ldots\ldots\ldots (8.1)$$

If an additive is present in the vessel at C_v and the contents of the vessel are subject to a dilution 'D' ($D = \frac{F}{V}$; Appendix 1.A) then the rate at which C_v will fall is :-

$$\frac{d\,C_v}{dt} = - \frac{C_v \times F}{V} \quad \ldots\ldots\ldots\ldots (8.2)$$

Thus the net rate of change of concentration is :-

$$\frac{d\,C_v}{dt} = \frac{F_{add} \times C_{add}}{V} - \frac{F \times C_v}{V} \quad \ldots\ldots (8.3)$$

If addition is commenced with no additive in the vessel ($C_v = 0$) then the additive concentration will increase in a negative exponential manner until the rate of addition approaches the rate of washout. That is when:-

$$\frac{F_{add} \times C_{add}}{V} = \frac{C_v \times F}{V} \quad \ldots\ldots\ldots (8.4)$$

Thus, for any value of C_v it is possible to calculate the exact concentration of additive needed in the infusate (C_{add}) to maintain C_v at a constant value, since F_{add}, V and F in equation 8.4 are known.

On this basis an experimental scheme using a series of increasing "steps" of fixed concentrations of additive in the culture, was devised. This was considered preferable to a regime where the additive concentration would be constantly changing in an irregular manner (ie a series of negative-exponential increments), as it would fully exploit the self-stabilising characteristics of the continuous culture system, by exposing the microflora to unchanging concentrations of additive for fixed periods of time.

Because of the dialysis system, any addition to the culture vessel would result in an actual concentration (C_p) in the vessel lower than the nominal "total" concentration : $\left[(C_d \times D_{fac}) + C_p\right]$. The ratio of solute in the dialysate to that in the culture; $(C_d \times D_{fac}) : C_p$ is virtually constant for a given value of D_{fac} and the range of low MW compounds being infused (Thomson 1974). Experiment showed that for F_d = 30 ml/h and F = 15 ml/h the ratio was approximately 1 : 1, thus the actual culture concentration would be approximately half the nominal total concentration.

The experimental procedure was as follows :- An initial 4 day culture period, to allow establishment of steady-state conditions, was followed by up to 6 days of additive infusion. The initial additive concentration was 0.025 M achieved by injection of an appropriate

amount of additive and maintained by an infusion of concentration calculated to equilibrate with a total nominal concentration of twice the above value. At 24 h intervals thereafter, additive concentration in the culture vessel was doubled by a further injection, and the infusion concentration adjusted accordingly.

Compounds Infused.

Infusion experiments were carried out with formic, acetic and propionic acids and with formaldehyde. Formaldehyde solutions were prepared from formalin (≈ 400 g/l formaldehyde). Organic acids were incorporated as their sodium salts to avoid excessive dilution rates arising from large deliveries of buffer solution. Appendix 1.C gives details of the amounts of additive compounds injected at each 24 h interval and the concentrations of infusate used during intervening periods. These figures assume an overall flow rate (F) of 15 ml/h $^{-1}$ (including substrate, buffer and additive infusion) and a culture volume of 450 ml. The desired flow rate was not achieved in practice and the concentrations of additive quoted are consequently nominal. To minimise changes in F, dialysis solution was delivered instead of infusate during the first period of each experiment. All additions to the culture vessel were taken into account in calculating the values of F_{fac} and D_{fac}, although certain components of the total (the additive injections) were added as discrete volumes rather than continuously.

The slight non-linearity introduced thereby was disregarded in the calculations.

Sampling and Analysis.

The culture was sampled on attaining a steady-state and at 24 h intervals thereafter, using the methods described in Section 7. The first two samples, before infusion commenced, were designated A_1 and A_2. Subsequent samples were numbered according to the infusion period from which they were drawn. Delivery rates of slurry, buffer and dialysate were recorded for each infusion period to allow calculations of the appropriate values of F_{fac} and D_{fac}. Differential counts, total counts, VFA analysis and NH_3-N determinations were carried out on each sample using the methods described in Section 4.

Substrate.

Details of the substrate silage are given in Table 14 (page 106). The slurry was prepared by the method already described (Appendix 2.M). Enough was produced in a single batch to meet the needs of all infusion experiments.

RESULTS.

1. Formic Acid Infusion.

Rumenstat data for the formic acid infusion experiment is given in Table 37; analytical results (corrected for F_{fac}) are presented in Table 38. Figure 42 shows TVFA and NH_3-N concentrations, and Figure 43, the concentrations of individual VFAs together with culture Eh, respectivly.

TVFA declined as the concentration of infused formate increased. This decline was associated with an increase in the proportion of butyrate in the TVFA. The concentration of NH_3-N also increased during the infusion period; a trend which was evident even when the figures are not corrected for F_{fac}. Differential counts of bacteria were apparently unaffected by the infusate although total counts were somewhat depressed (Table 38). The removal of F_{fac} from these figures shows that the measured values fell.

2. Acetic Acid Infusion.

Rumenstat data for the acetic acid infusion experiment is given in Table 39. Table 40 gives the analytical results corrected for F_{fac}. TVFA, TVFA other than acetate and NH_3-N are shown in Figure 44; the individual VFA concentrations and culture Eh are shown in Figure 45. This infusion was terminated at period 5 when

Table 37. Rumenstat data; formic acid infusion - S 37 silage.

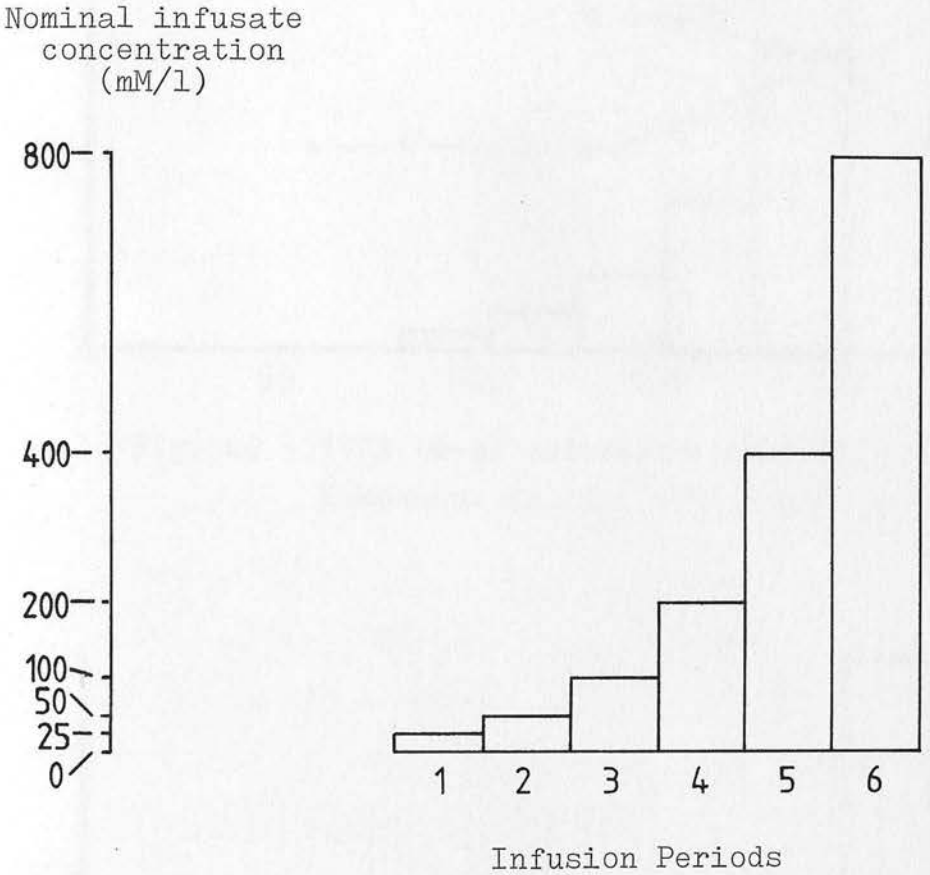
Period :	INITIAL, $[A_1 + A_2]$	1	2	3	4	5	6	Totals
time (h)	84	24	24	24	24	24	24	230
[C] additive (mM/l)	0	25	50	100	200	400	800	-
V_{add} (infused)	84	24	24	24	24	24	24	228
V_{add} (injected)	0	4	4	8	16	30	55	117
V_b (ml)	220	50	60	60	70	70	80	610
V_f (ml)	866	248	248	248	248	248	248	2354
V (ml)	1170	326	336	340	358	372	407	3309
F (ml/h)	13.929	13.58	14.00	14.12	14.92	15.50	16.96	14.387
F_{fac} / F_f †	1.351	1.315	1.355	1.371	1.444	1.500	1.641	10.304 †
V_d	2410	830	830	830	830	830	830	7960
D_{fac} / F_d ▽	2.376	2.437	2.364	2.344	2.218	2.135	1.952	33.1 ▽

Table 38. Analytical results (corrected for F_{fac}) with formate infusion into Rumenstat culture of S 37 silage.

Period:	A_1	A_2	1	2	3	4	5	6
TVFA (mM/l)	122	120	112	90.4	88.7	83.7	72.8	52.9
M % acetate	70.6	68.4	69.4	69.3	66.0	66.7	66.6	61.4
M % propionate	14.0	15.1	13.6	11.5	13.3	11.1	9.2	13.3
M % butyrate	9.5	10.2	10.8	11.7	13.0	13.6	16.0	13.4
NH_3-N (mg/l)	112	115	112	112	123	149	164	187
log organisms/ ml (starch)	-	5.22	5.17	5.10	5.12	5.47	5.19	-
log organisms/ ml (gelatin)	-	5.48	6.23	5.26	5.30	6.45	5.24	-
log organisms/ ml (cellulose)	-	5.14	6.17	6.06	6.17	6.37	6.35	-
log total bacteria/ ml	-	8.16	8.17	8.30	8.16	8.02	7.99	7.99
log total protozoa/ ml	-	4.22	4.33	4.30	4.37	4.21	4.21	4.34

IN VITRO INFUSION EXPERIMENTS

Explanation of overlays to Figures 42-49



The legends are ommitted from the overlays for clarity.

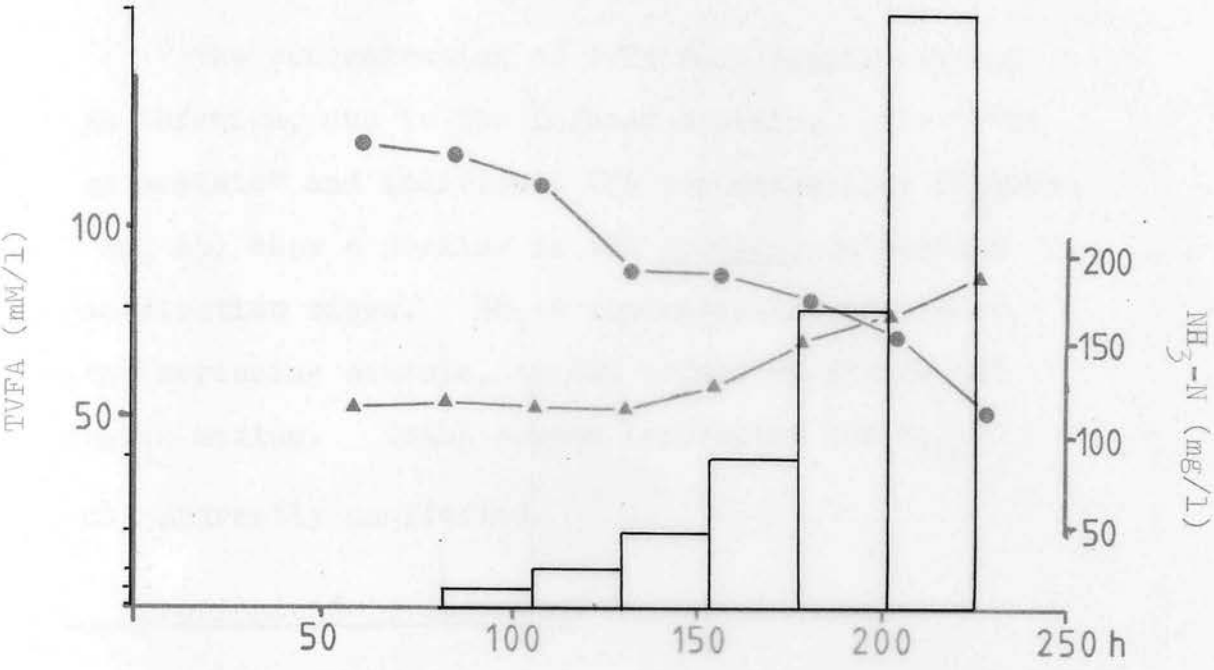


Fig. 42 TVFA (●-●) and NH_3-N (▲-▲) in Rumenstat culture with formic acid infusion.

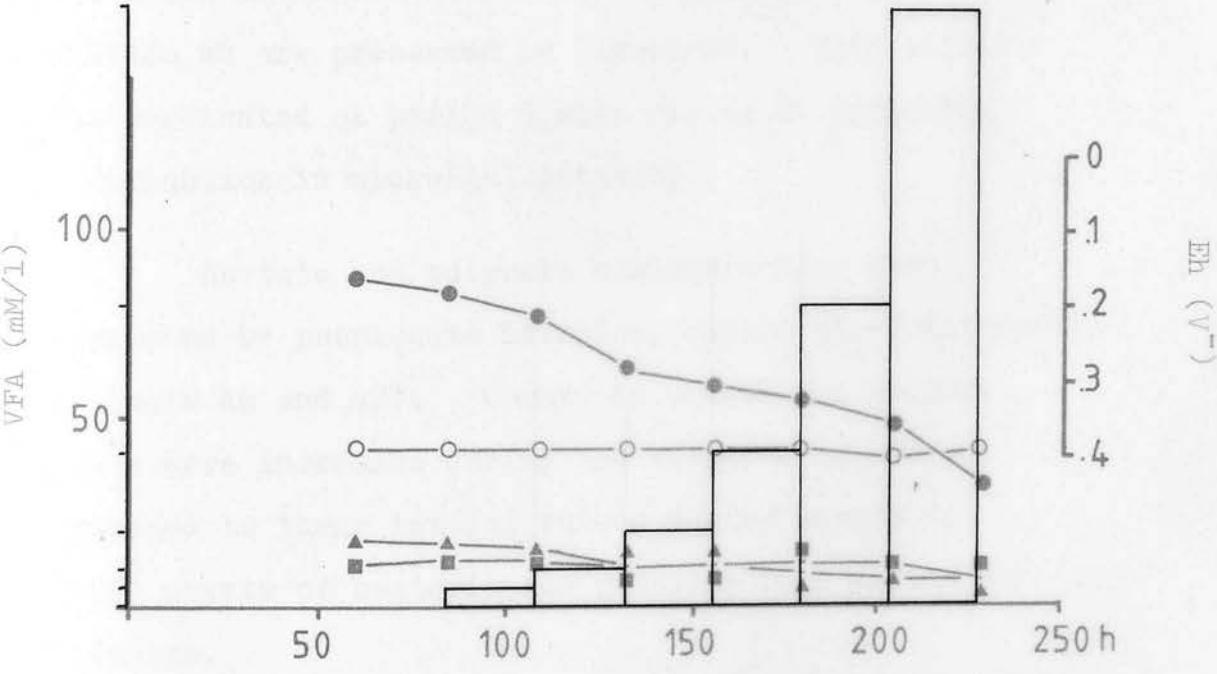
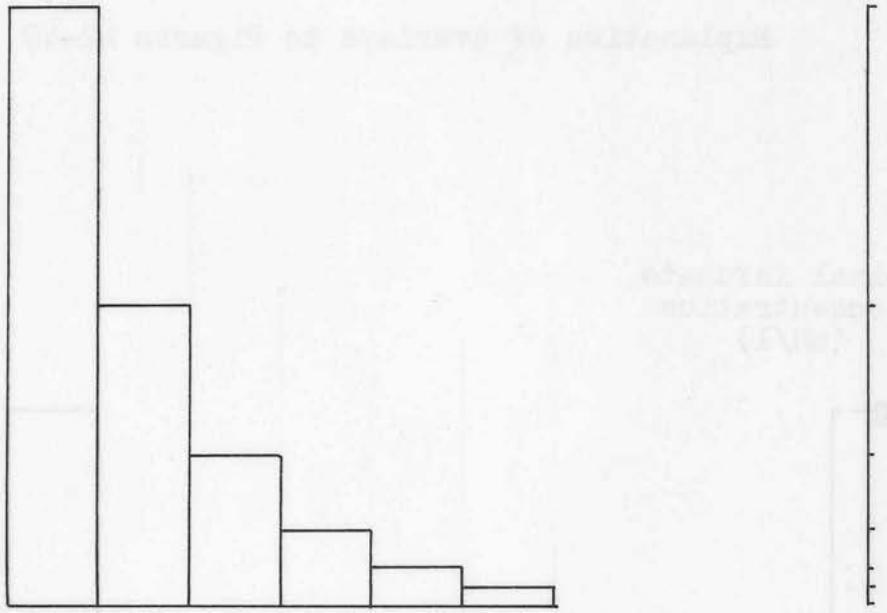
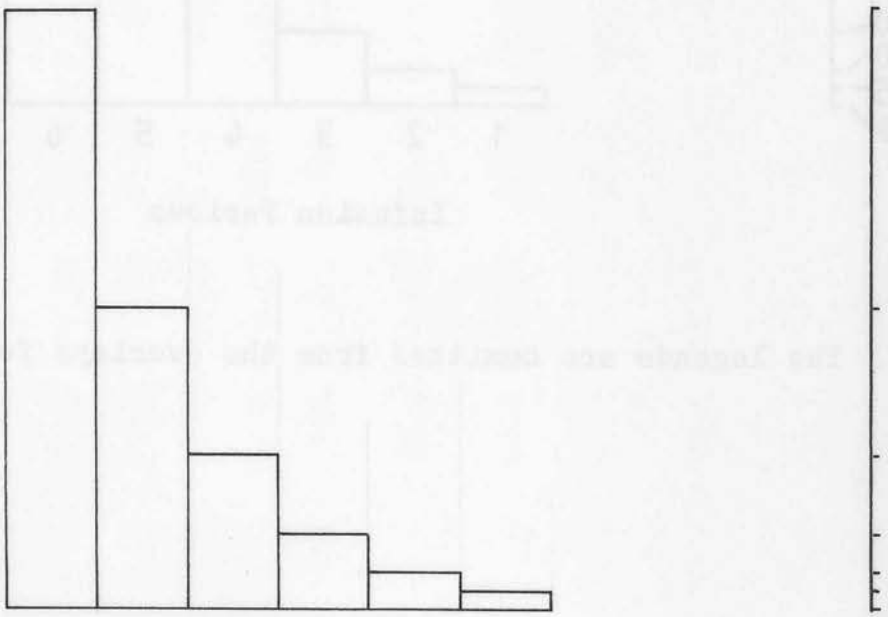


Fig. 43 Acetic acid (●-●), propionic acid (▲-▲) butyric acid (■-■) and Eh (o-o) in Rumenstat culture with formic acid infusion.





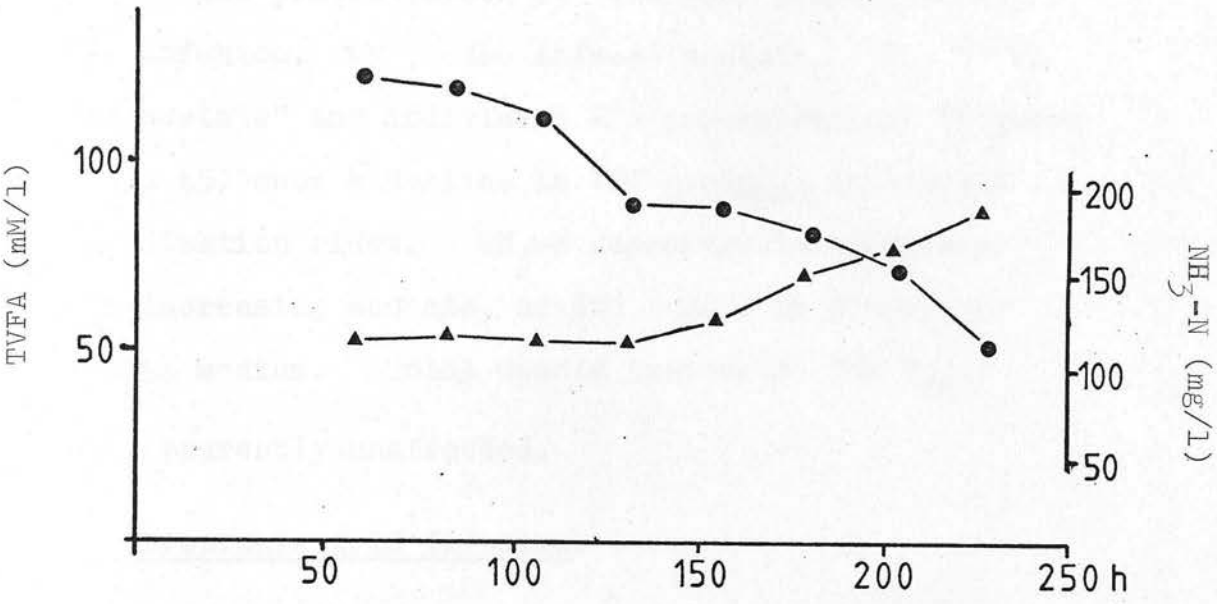


Fig. 42 TVFA (●-●) and NH_3-N (▲-▲) in Rumenstat culture with formic acid infusion.

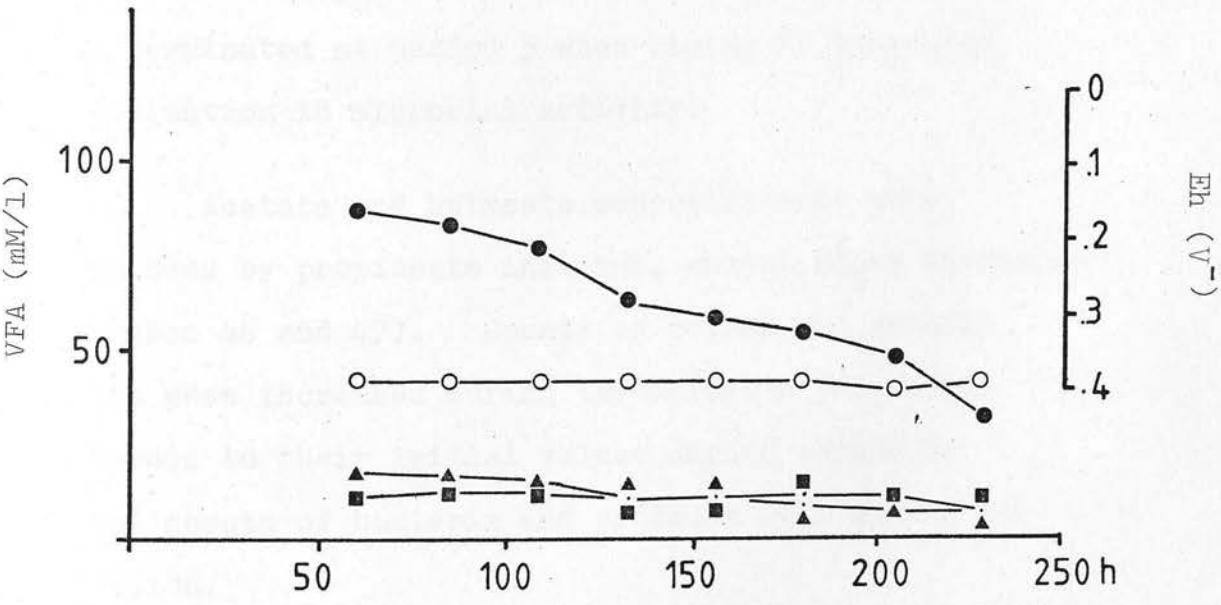


Fig. 43 Acetic acid (●-●), propionic acid (▲-▲) butyric acid (■-■) and Eh (o-o) in Rumenstat culture with formic acid infusion.

rising Eh indicated deminishing microbial activity.

The concentration of TVFA rose rapidly during this infusion, due to the infused acetate. The "TVFA less acetate" and individual VFA concentrations (Figures 44 and 45) show a decline in VFA produced as acetate concentration rises. NH_3-N concentration increased with increasing acetate, as did counts on starch and gelatin medium. Total counts (corrected for F_{fac}) were apparently unaffected.

3. Propionic Acid Infusion.

Tables 41 and 42 give Rumenstat and analytical data for the infusion experiments with propionic acid. The values of TVFA, "TVFA less propionate" and NH_3-N are shown in Figure 46 and the individual VFAs and culture Eh are presented in Figure 47. This infusion was terminated at period 5 when rising Eh indicated a diminution in microbial activity.

Acetate and butyrate concentrations were depressed by propionate infusion, whilst NH_3-N increased (Figures 46 and 47). Counts on starch and gelatin media were increased during the infusion period but returned to their initial values during period 5. Total counts of bacteria and protozoa fell during the infusion.

Table 39. Rumenstat data; acetic acid infusion - S 37 silage.

Period :	Initial, $[A_1 + A_2]$	1	2	3	4	5	Totals
time (h)	97	24	24	24	24	24	217
[C] additive (mM/l)	0	25	50	100	200	400	-
V_{add} (infused)	97	24	24	24	24	24	217
V_{add} (injected)	0	5	5	10	20	40	80
V_b (ml)	190	50	50	60	60	70	480
V_f (ml)	895	220	220	220	220	220	1995
V (ml)	1182	299	299	314	324	354	2772
F (ml/h)	12.186	12.458	12.458	13.080	13.500	14.750	12.774
F_{fac} / F_f^{∇}	1.321	1.359	1.359	1.427	1.45	1.61	9.217^{∇}
V_d	2645	655	655	655	655	655	5920
D_{fac} / F_d^{Δ}	2.239	2.189	2.189	2.086	2.021	1.849	27.28^{Δ}

Table 40. Analytical results (corrected for F_{fac}) with acetate infusion into Rumenstat culture of S 37 silage.

Period :	A_1	A_2	1	2	3	4	5
TVFA (mM/l)	126	123	116	136	180	≈ 215	> 400
M % acetate	68.7	70.3	70.8	78.4	90	≈ 95	≈ 99
M % propionate	15.3	14.6	13.8	8.8	3.5	2.0	< 1
M % butyrate	10.3	9.9	10.5	9.0	4.4	3.1	< 1
NH_3-N (mg/l)	91.3	134	142	148	142	175	207
log organisms/ ml (starch)	-	5.46	5.23	5.24	6.11	7.13	-
log organisms/ ml (gelatin)	-	5.34	5.31	4.99	6.20	7.11	-
log organisms/ ml (cellulose)	-	5.10	5.07	5.22	4.93	4.97	-
log total bacteria/ ml	8.31	8.19	8.31	7.99	8.12	7.95	7.95
log total protozoa/ ml	4.16	4.15	4.10	3.98	4.02	4.10	3.26

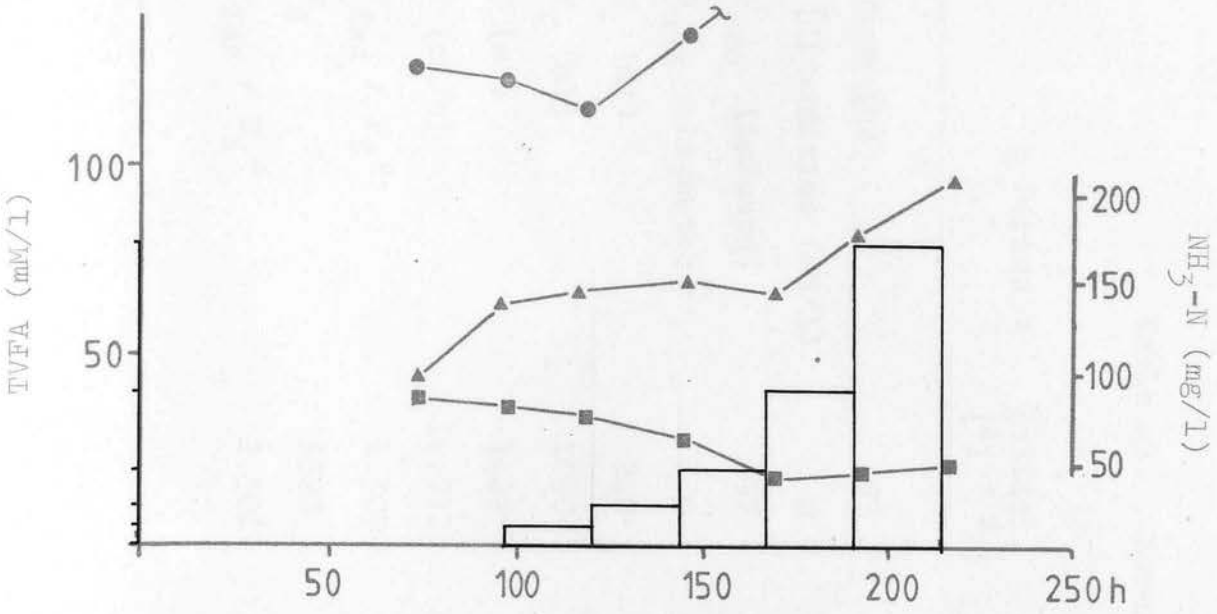


Fig. 44 TVFA (●-●), TVFA other than acetate (■-■) and NH_3-N (▲-▲) in Rumenstat culture with acetic acid infusion.

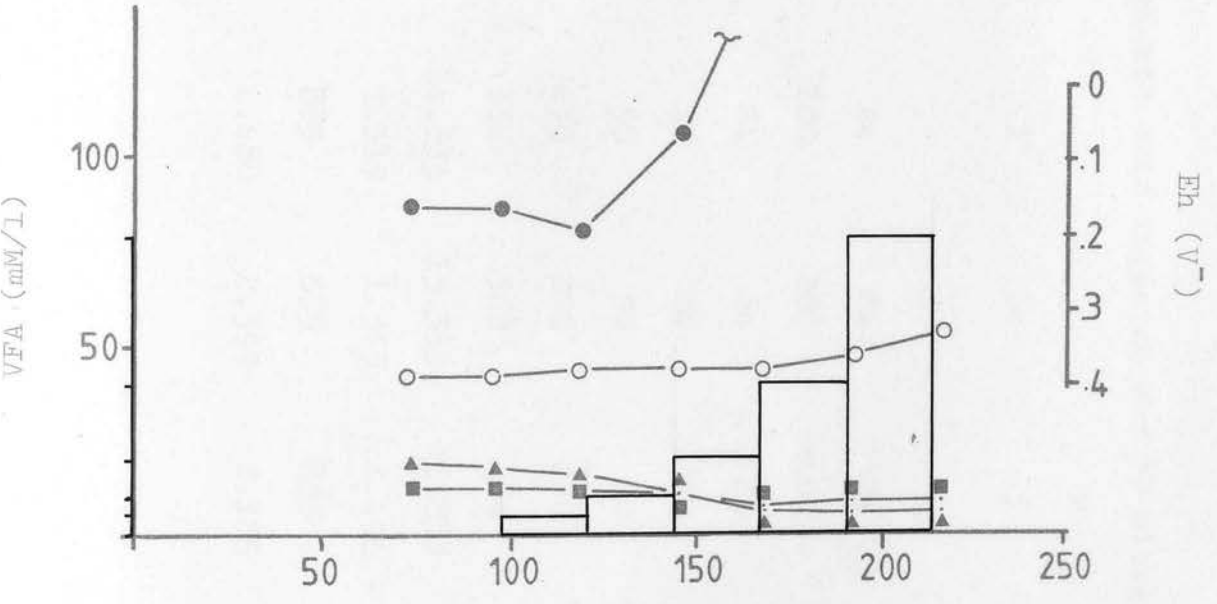
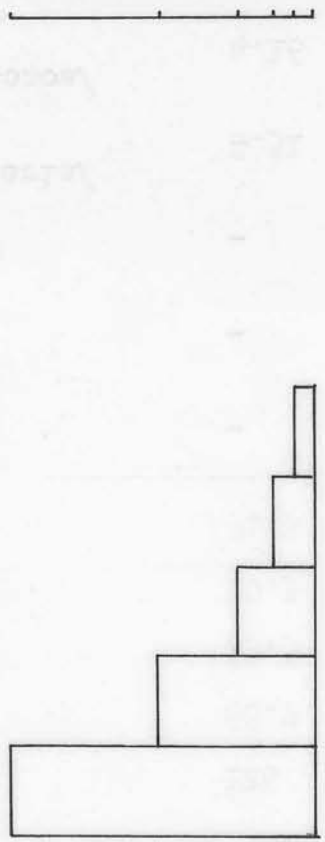
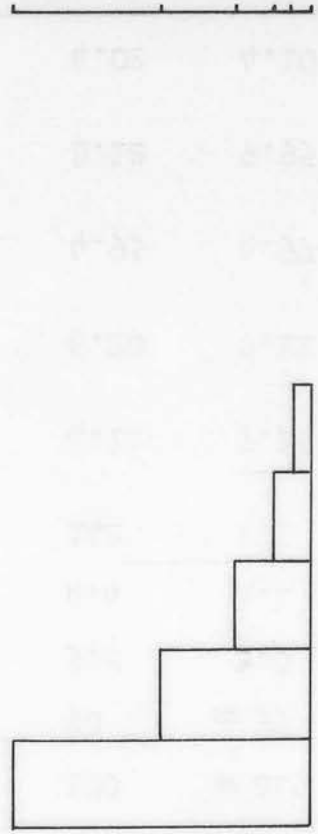


Fig. 45 Acetic acid (●-●), propionic acid (▲-▲), butyric acid (■-■) and Eh (o-o) in Rumenstat culture with acetic acid infusion.





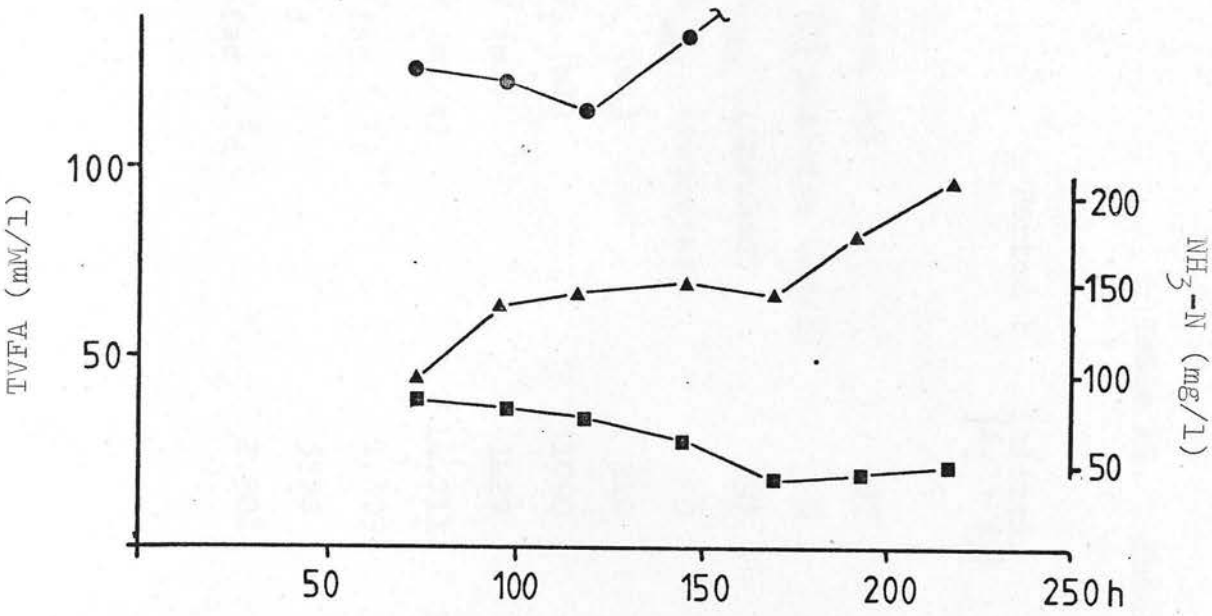


Fig. 44 TVFA (●-●), TVFA other than acetate (■-■) and NH_3-N (▲-▲) in Rumenstat culture with acetic acid infusion.

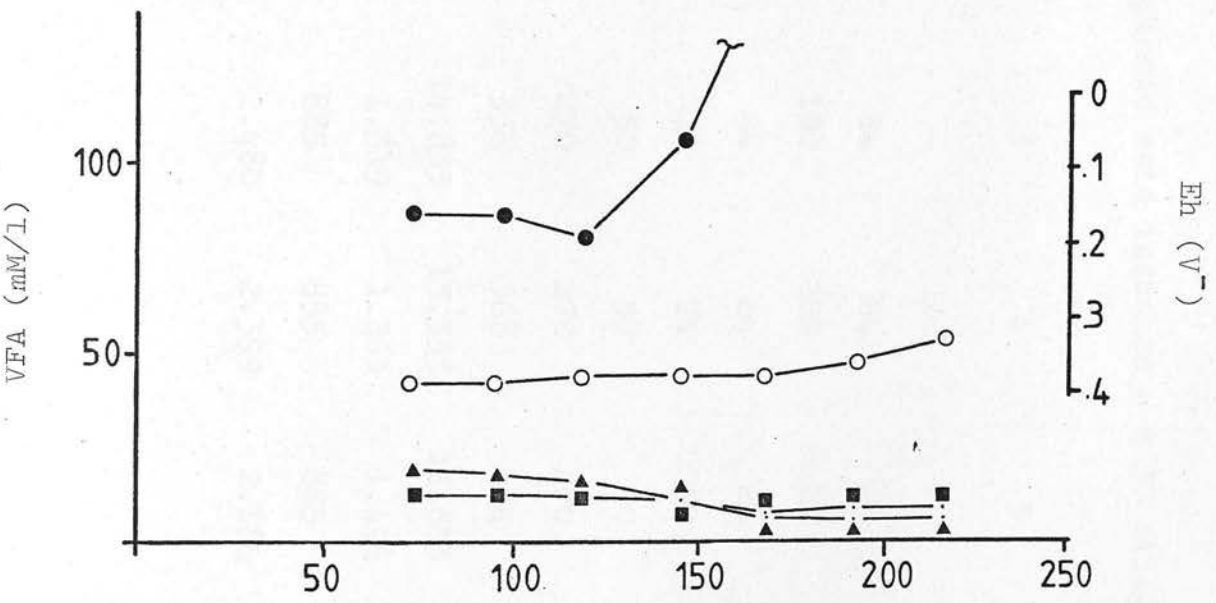


Fig. 45 Acetic acid (●-●), propionic acid (▲-▲), butyric acid (■-■) and Eh (o-o) in Rumenstat culture with acetic acid infusion.

Table 41. Rumenstat data; propionic acid infusion - S 37 silage.

Period :	Initial, $[A_1 + A_2]$	1	2	3	4	5	Totals
time (h)	97	24	24	24	24	24	217
[C] additive (mM/l)	0	25	50	100	200	400	-
V_{add} (infused)	97	24	24	24	24	24	217
V_{add} (injected)	0	6	6	12	24	50	98
V_b (ml)	240	50	40	50	50	60	490
V_f (ml)	1090	270	270	270	270	270	2440
V (ml)	1427	350	340	356	368	404	3245
F (ml/h)	14.711	14.583	14.167	14.833	15.333	16.833	14.954
F_{fac} / F_f^{∇}	1.309	1.296	1.259	1.319	1.363	1.496	11.25^{∇}
V_d	3570	885	885	885	885	885	7910
D_{fac} / F_d^{Δ}	2.501	2.523	2.597	2.480	2.399	2.186	36.79^{Δ}

Table 42. Analytical results (corrected for F_{fac}) with propionate infusion into Rumenstat culture of S 37 silage.

Period :	A_1	A_2	1	2	3	4	5
TVFA (mM/l)	127	127	135	136	177	≈300	>400
M % acetate	70.8	72.5	62.5	44.9	31.4	≈15	≈5
M % propionate	15.1	14.9	29.6	46.5	61.1	≈90	≈95
M % butyrate	9.3	8.3	5.86	4.54	3.02	2	<1
NH_3-N (mg/l)	103	117	116	108	127	154	200
log organisms/ ml (starch)	5.55	5.84	6.61	7.43	-	7.78	5.98
log organisms/ ml (gelatin)	5.37	5.47	6.63	6.52	-	7.03	5.71
log organisms/ ml (cellulose)	4.90	5.02	5.17	4.89	-	5.94	5.10
log total bacteria/ ml	8.29	8.17	8.14	8.09	7.65	7.66	7.48
log total protozoa/ ml	4.31	4.02	3.99	4.06	4.04	3.99	3.91

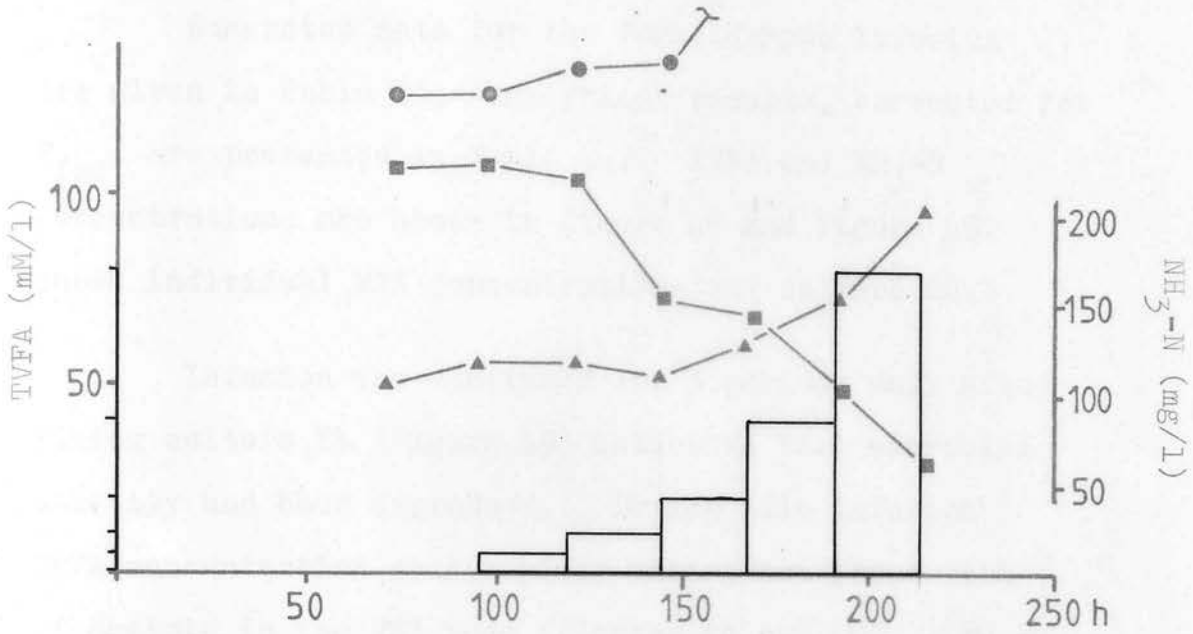


Fig. 46 TVFA (●-●), TVFA other than propionate (■-■) and NH_3-N (▲-▲) in Rumenstat culture with propionic acid infusion.

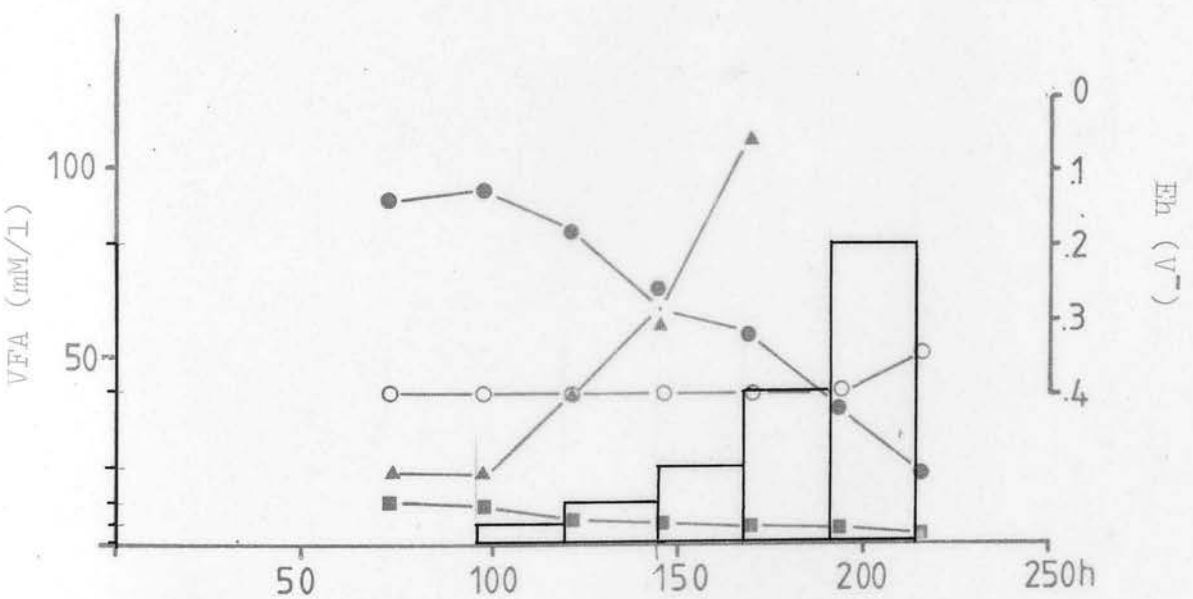
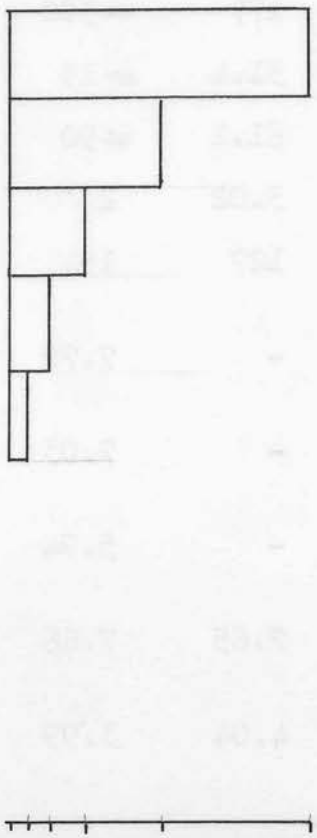
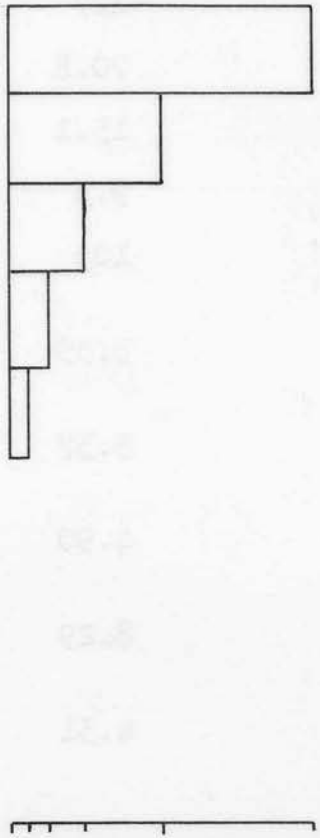


Fig. 47 Acetic acid (●-●), propionic acid (▲-▲), butyric acid (■-■) and Eh (o-o) in Rumenstat culture with propionic acid infusion.





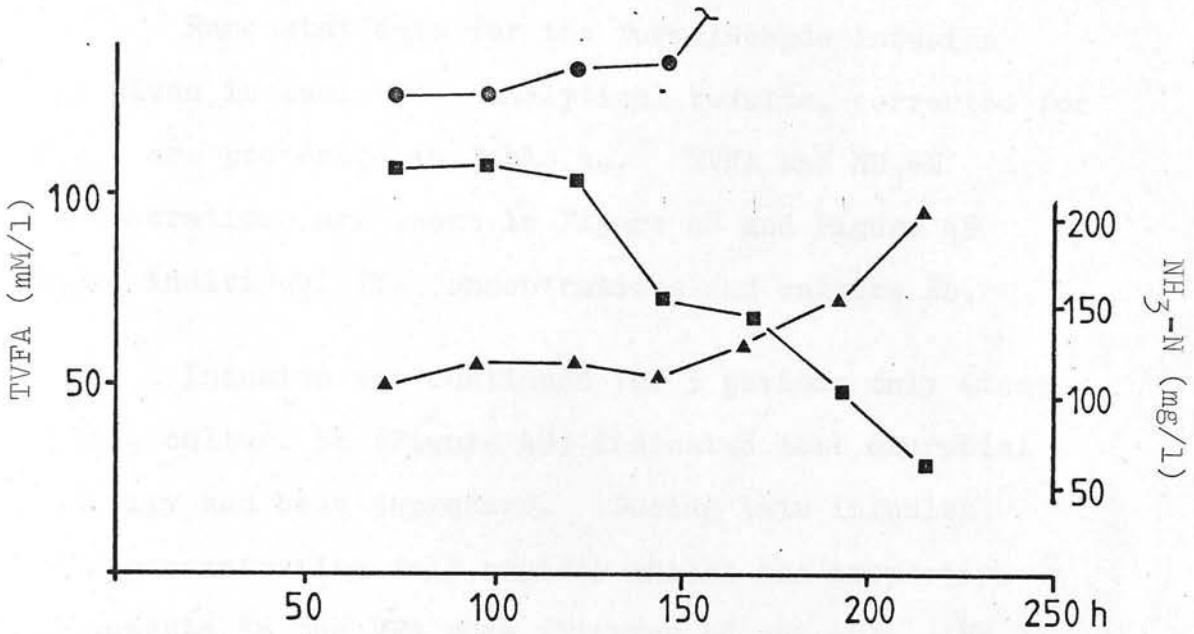


Fig. 46 TVFA (●-●), TVFA other than propionate (■-■) and NH_3-N (▲-▲) in Rumenstat culture with propionic acid infusion.

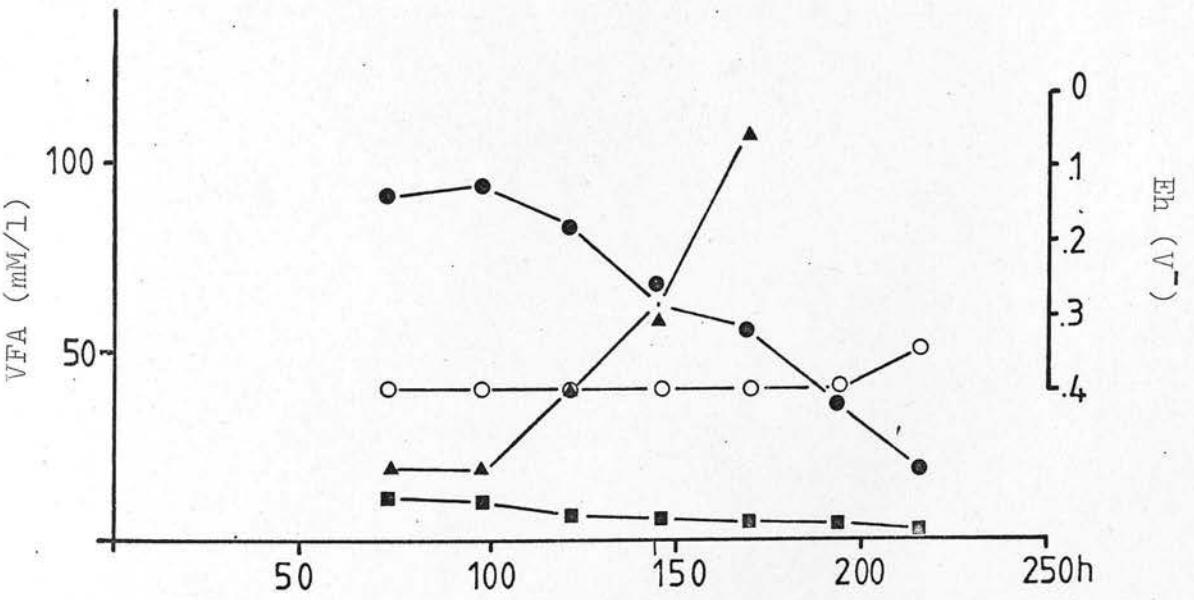


Fig. 47 Acetic acid (●-●), propionic acid (▲-▲), butyric acid (■-■) and Eh (o-o) in Rumenstat culture with propionic acid infusion.

4. Formaldehyde Infusion.

Rumenstat data for the formaldehyde infusion are given in Table 43; analytical results, corrected for F_{fac}, are presented in Table 44. TVFA and NH_3-N concentrations are shown in Figure 48 and Figure 49 shows individual VFA concentrations and culture Eh.

Infusion was continued for 3 periods only since rising culture Eh (Figure 49) indicated that microbial activity had been depressed. During this infusion TVFA concentration fell rapidly whilst the proportion of acetate in the VFA rose (Figures 48 and 49). NH_3-N and microbial counts fell from period 1 of this infusion although the total bacterial count was depressed less markedly than the differential counts.

Table 43. Rumenstat data; formaldehyde infusion - S 37 silage.

Period :	Initial, $[A_1 + A_2]$	1	2	3	4	Totals
time (h)	98	24	24	24	24	194
[C] additive (mM/l)	0	25	50	100	200	-
V_{add} (infused)	98	24	24	24	24	194
V_{add} (injected)	0	2	2	4	8	16
V_b (ml)	240	40	40	30	10	360
V_f (ml)	1030	250	250	250	250	2030
V (ml)	1368	316	316	308	292	2600
F (ml/h)	13.959	13.167	13.167	12.833	12.167	13.402
F_{fac} / F_f^{∇}	1.328	1.264	1.264	1.232	1.168	10.45^{∇}
V_d	3200	785	785	785	785	6340
D_{fac} / F_d^{Δ}	2.341	2.482	2.482	2.547	2.686	32.68^{Δ}

Table 44. Analytical results (corrected for F_{fac}) with formaldehyde infusion into Rumenstat culture of S 37 silage.

Period :	A_1	A_2	1	2	3
TVFA (mM/l)	126	129	47.5	24.4	16.6
M % acetate	70.4	71.4	70.9	74.9	80.9
M % propionate	15.7	16.1	15.8	12.5	2.5
M % butyrate	8.5	7.5	6.90	6.5	6.13
NH_3-N (mg/l)	116	126	76.9	17.6	4.63
log organisms/ ml (starch)	-	6.91	6.93	5.29	4.00
log organisms/ ml (gelatin)	-	6.50	6.30	6.96	4.43
log organisms/ ml (cellulose)	-	6.36	5.24	4.51	4.40
log total bacteria/ ml	-	8.34	8.27	8.39	7.20
log total protozoa/ ml	-	4.12	4.08	4.02	3.29

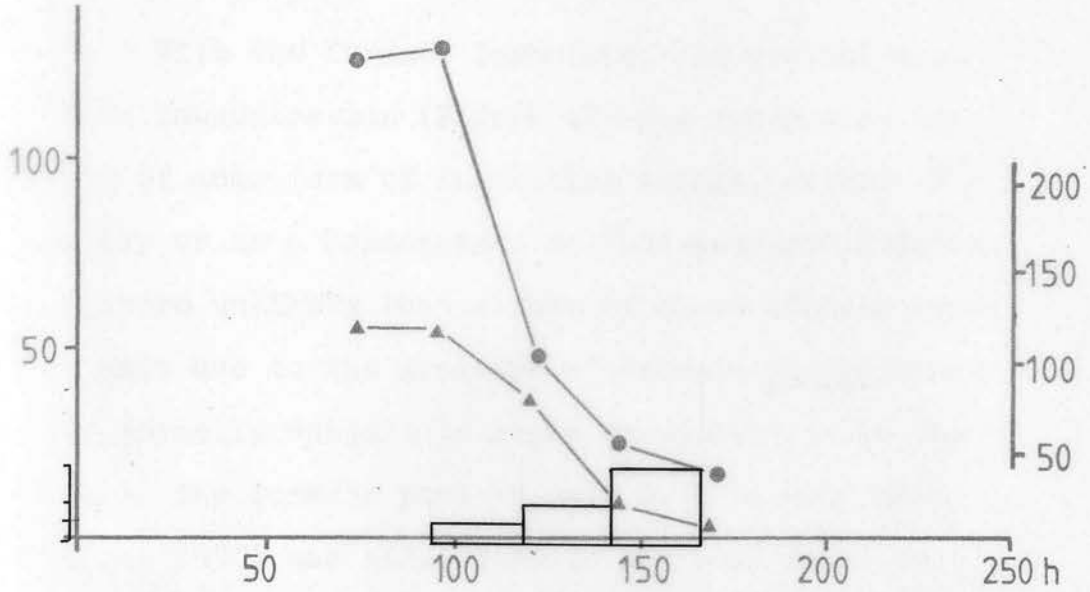


Fig. 48 TVFA (●-●) and NH_3-N (▲-▲) in Rumenstat culture with formaldehyde infusion.

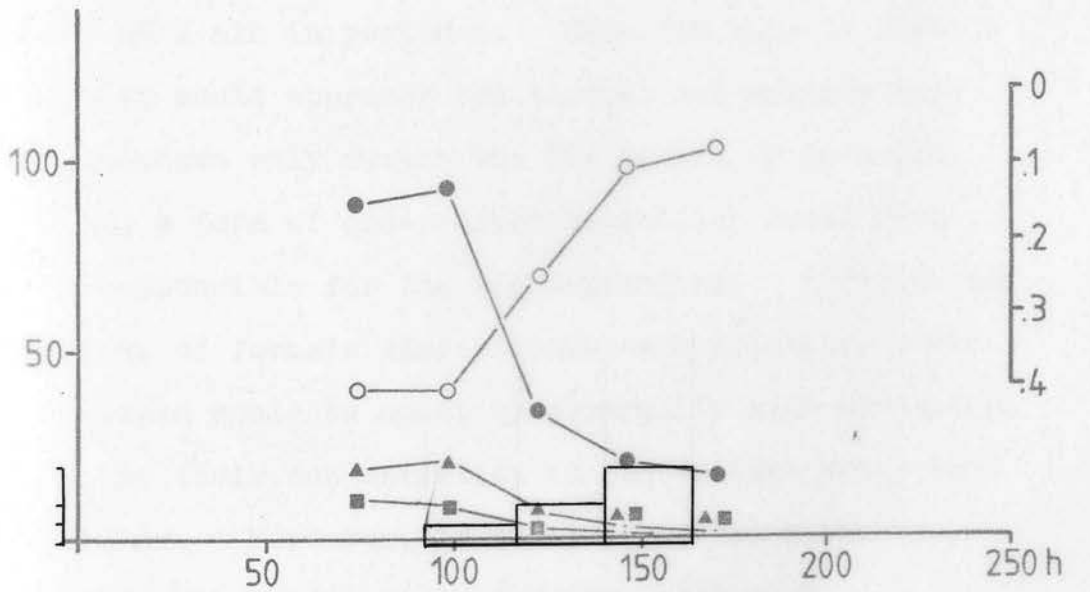


Fig. 49 Acetic acid (●-●), propionic acid (▲-▲), butyric acid (■-■) and Eh (o-o) in Rumenstat culture with formaldehyde infusion

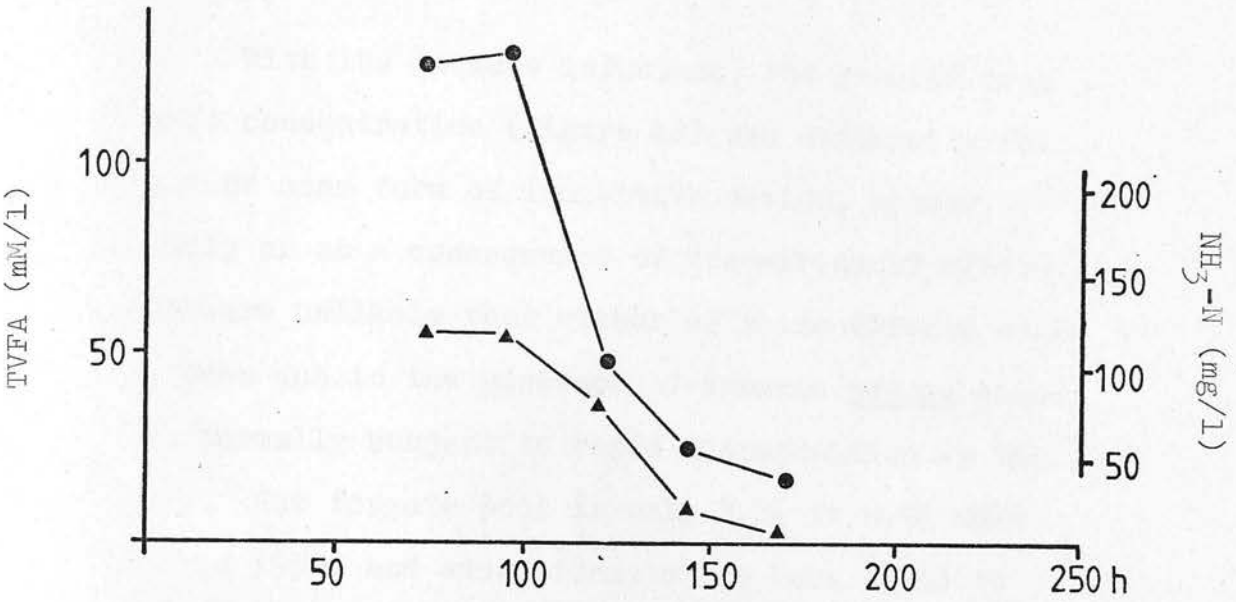


Fig. 48 TVFA (●-●) and NH_3-N (▲-▲) in Rumenstat culture with formaldehyde infusion.

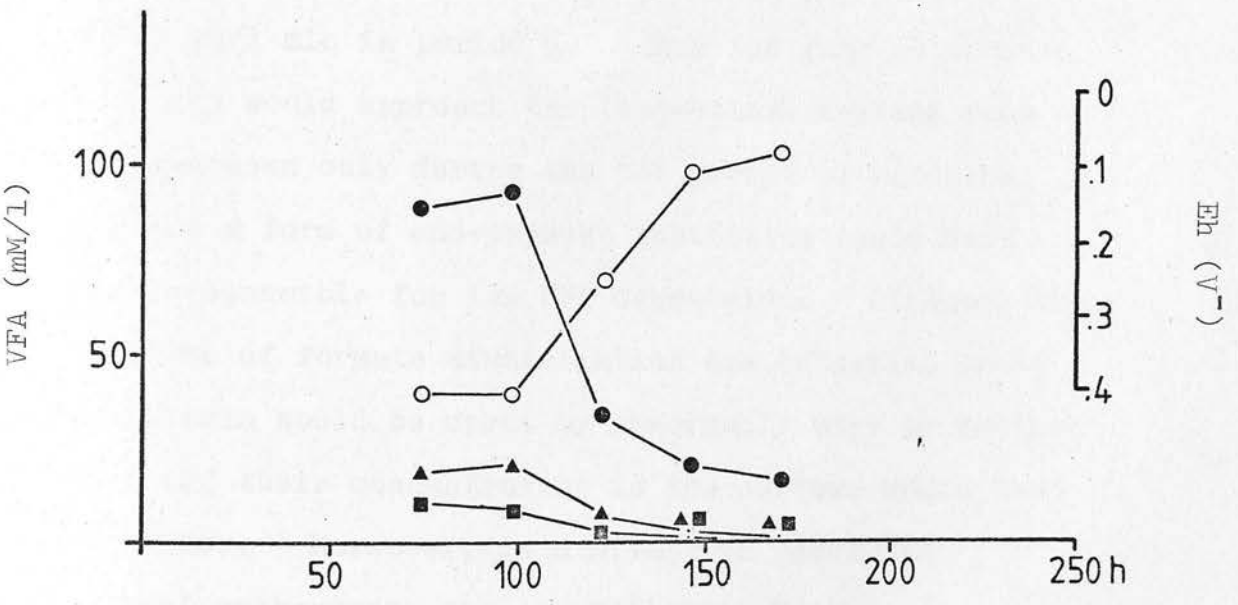


Fig. 49 Acetic acid (●-●), propionic acid (▲-▲), butyric acid (■-■) and Eh (o-o) in Rumenstat culture with formaldehyde infusion

DISCUSSION

With the formate infusions, the gradual drop in TVFA concentration (Figure 42) was apparently the result of some form of inhibitive action, either directly or as a consequence of "end-product" effects. It appears unlikely that either of these effects could have been due to the presence of formate *per se* since it is normally subject to rapid decomposition in the rumen. The formate pool is only 0.01 to 0.02 mM/l (Hungate 1950) and added formate has been found to disappear at 0.628 $\pm$ 0.33 mM/l min, a rate considered to be the maximum capacity for formate metabolism (Carroll and Hungate 1955). Rates of infusion in these experiments (gross rates, disregarding the effects of dialysis) ranged from 0.03 mM/l min in period 1 to 0.975 mM/l min in period 6. Thus the rate of formate addition would approach the theoretical maximum rate of breakdown only during the 5th period of infusion. However a form of end-product inhibition could have been responsible for the VFA depression. Although the products of formate dissimilation are volatile, their equilibria would be upset by abnormally high production rate and their concentration in the culture would thus be higher. Moreover, in a situation where the ruminal methanogens such as *Methanobacterium ruminantium* are saturated with formate as substrate,

their alternative substrates, carbon dioxide and hydrogen (Smith and Hungate 1958), will be much less rapidly utilised. The pathways leading from pyruvate to acetate and butyrate supply carbon dioxide and hydrogen, which are utilised by the methanogens. Energy production *via* acetate and butyrate will thus be metabolically blocked. The fall in acetate observed during the formate infusion may have largely been due to this.

The observed rise in NH_3-N concentration, which occured together with the fall in acetate and other VFA discussed above, almost certainly reflects a suppression of microbial energy metabolism. This rise could have been due to failure of the microflora, in conditions of increasing energy deficit, to utilise available nitrogen for synthetic purposes. Alternatively the blocking of VFA pathways may have caused an increase in the use of nitrogenous compounds as energy sources, resulting in the production of ammonia as a terminal metabolite. The increasing proportions of *iso* acids in the VFA (Appendix 3.C.1) suggest that this latter mechanism was at least partly responsible for the pattern of change in vitro NH_3-N.

With acetate and propionate infusions the concentrations of other VFA fell as infusate

concentration increased (Figures 44 and 46). The reductions in propionic acid and butyric acid concentrations over five days of acetate infusion (18.6 to 6.3 and 12.6 to 9.7 mM/l respectively) were close to those when formate was infused (18.0 to 7.0 and 12.1 to 7.1 mM/l). This suggests that similar mechanisms of inhibition were functioning. It was not possible to estimate how much acetic acid was produced by fermentation during infusion since the exact equilibrium concentrations across the dialysis membranes are unknown and the higher infusate concentrations were outwith the range of accurate determination.

In the case of propionate infusion, the fall in TVFA produced was greater than that resulting from formate infusion (TVFA other than propionate was down to approximately 29 mM/l in period 5 when propionate was infused compared with 46 mM/l in period 6 when formate was infused). This probably reflects the greater persistence of propionate in the culture but may also reflect a more inhibitive effect of a compound which is a higher homologue (Czerkawski 1969). The greater inhibition of butyric acid production by propionate than acetate (2.6 mM/l in period 5 compared with 7.0 mM/l) accords with the theory of higher homologue effect as well as the more general proposal

that these are end-product inhibition phenomena.

The similarities of NH_3-N patterns with acetate and propionate infusions, to that with formate (Figures 42, 44 and 46) further suggests that the general consequences of inhibition were the same in each case ie. microbial energy metabolism was depressed. The observation of increased ruminal NH_3-N with VFA infusions appears to be in conflict with the findings of Atwal, Milligan and Young (1974) who showed that the addition of VFA to high protein supplements administered *via* rumen fistulae, reduced the ruminal NH_3-N concentration, compared with untreated protein supplements. These experiments of Atwal *et al*.were based on VFA treatment of the protein and not direct infusion of free acids or salts. The authors do not offer any biochemical explanation of the mode of action of the VFA and suggest that a direct bacteriostatic effect, in the vicinity of the protein particles, was responsible.

The effects of formaldehyde infusion were considerably more marked than those of any of the organic acid salts. Whilst the latter did not bring about a rise in culture Eh until period 5 when the infusate concentration was nominally 400 mM/l (Figures 43, 45 and 47), only 25 mM/l of formaldehyde in period 1 resulted in substantial falls in TVFA, NH_3-N and culture Eh. Each of these parameters was similarly affected, apparently as a consequence of simple inhibition.

More worthwhile data might have been obtained if lower initial concentrations of formaldehyde had been used.

The concentrations of formaldehyde and organic acids applied as silage additives in practice are of the same order of magnitude and the considerable differences in their effects are thus important. Woolford (1975) showed that, in pure culture, a concentration of 8 mM/l free formaldehyde was sufficient to supress a wide range of microorganisms. There is no reason to suppose that the inhibitory concentration for rumen bacteria will be significantly greater than this, although in ruminal culture actual inhibitory concentrations will be somewhat higher, owing to the presence of organic matter. During ensilage the proportion of formaldehyde remaining falls, (Wilson 1976) whereas in the present experiment there was free formaldehyde at all times. This aspect of the antimicrobial action of formaldehyde should be borne in mind when devising and interpreting experiments of this type.

Microbial inhibitors in the rumen - Reprise.

Ruminal methanogenesis is known to be reduced by the administration of unsaturated higher

fatty acids (Czerkawski 1969). This has been explained in terms of the provision of an alternative acceptor of metabolic hydrogen, although the effect on methanogenesis is greater than can be accounted for by complete saturation of the available double bonds in unsaturated acids. Higher saturated fatty acids also bring about this effect, but to a lesser extent and in both cases an increase occurs in the proportion of propionate in the VFA. The production of propionate consumes metabolic hydrogen, but this cannot completely explain the reduction of methane production in the presence of fatty acids. The enhanced effect of unsaturated fatty acids could partly be due to their different physical properties. At rumen temperatures the higher fatty acids are solids (eg. stearic acid - MP 69°C, palmitic acid - MP 64°C) whereas unsaturated acids are liquid (eg. oleic acid - MP 14°C). The unsaturated acids may be more easily dispersed and consequently come in contact more readily with microbial cells.

It is possible that fatty acids act by simple toxicity; the reduction in cellulolysis observed along with depressed methanogenesis in the experiments of Czerkawski et al. 1966 [a,b,c] is consistent with this explanation. The toxicity theory would also explain

the observation of increased proportions of propionate which could be a consequence of reduced acetate production from cellulolysis.

Rumen methanogens are Gram +ve and thus more susceptible to the action of fatty acids than are most other rumen organisms which are Gram -ve or Gram variable (Nieman 1954). The fact that propionate producers are apparently much less inhibited by fatty acids than other ruminal organisms (i.e., the toxicity is selective) suggests that these compounds may not act as general inhibitors. In these infusion experiments formaldehyde, which is non-selective in action, depressed all VFA production to a similar extent. With the organic acid salts the expected end-product inhibition occured, acetate being especially inhibited. Actual mechanisms of end-product inhibition in the rumen are difficult to describe in definitive terms, since the point of action may be remote from the observed effect, as was the case with the formate infusions. There is some evidence in the present results which suggests that this mechanism may play a part in inhibitive effects of the higher fatty acids.

Microbiology of infusion experiments.

Total counts of microorganisms did not alter significantly during infusion experiments with organic

acids although a ten-fold fall in both total bacterial and protozoal numbers occured durin the last period of formaldehyde infusion. The total-count technique does not allow discrimination between living and dead cells. However, since a continuous culture constantly washes out cells, growth must match flow rate for numbers to be maintained (Appendix 1.A) and a fall in total-count means de facto a fall in numbers of viable organisms.

Only with the formaldehyde infusion did viable counts fall. Rate of fall varied for the different selective media (Figure 50). Interpretation of these observations is difficult since microbial numbers do not necessarily reflect the extent of their biological activity (Hobson 1972) and a change in the estimated numbers of any one type by a factor of 10 or less is probably of marginal significance in a complex mixed population such as that of the rumen.

Implications for the additive treatment of silages.

The organic acid silage additives used at normal application rates ($\leq$ 5 kg/tonne) would be unlikely to result in concentrations greater than 25 mM/l in the rumen and under most practical circumstances the concentration would be much lower than this. The present results suggest that only a slight disturbance of ruminal microbiology and

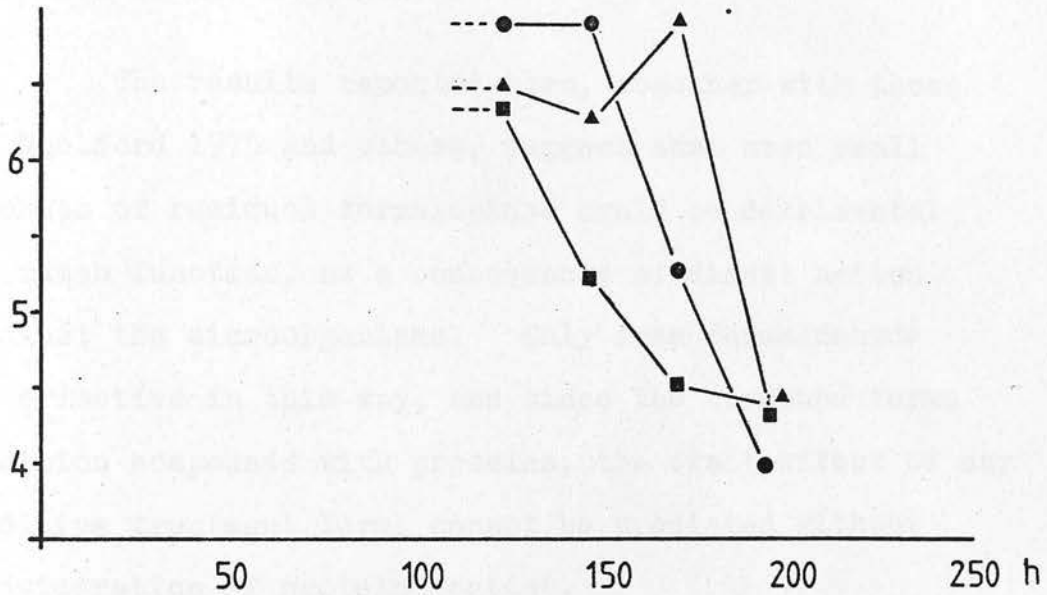


Fig. 50 Differential bacterial counts on starch medium (●-●), gelatin medium (▲-▲) and cellulose medium (■-■) during formaldehyde infusion.

biochemistry would occur as a consequence of such additive treatment and even at higher levels of application the effects in physiological terms would not be serious.

Formaldehyde is a special case, differing both chemically, and in its mode of action, from the organic acids. It is the only sterilant in regular commercial use as a silage additive.

The results reported here, together with those of Woolford 1975 and others, suggest that even small amounts of residual formaldehdye could be detrimental to rumen function, as a consequence of direct action against the microorganisms. Only free formaldehyde is effective in this way, and since the compound forms addition compounds with proteins, the exact effect of any additive treatment level cannot be predicted without consideration of protein content.

GENERAL DISCUSSION

THE IN VITRO RUMEN.

The work described in this thesis was primarily concerned with evaluating the effects of silage additives in the rumen. The adoption of in vitro techniques was coincidental to this objective but necessitated intensive persual of the practice and technology of in vitro systems. This technology is still in a formative phase and several developments arose from the present work. Some of the innovations described here, such as the adoption of a discrete "in culture" dialysis system or the more prosaic belt-driven magnetic stirrers were also introduced in concurrent research by other workers. Abe and Kumeno (1973) employed a dialysis system similar to that desribed here and the fermenter of Slyter (1975) used belt-driven stirrers, albeit some eleven years after this author first published an in vitro design (Slyter et al., 1964). The use of automatic pH control was adopted for the apparatus described by Slyter (1975), but the earliest example of this, apart from the use of ion exchange resins by Rufener (1963), was that of Aafjes and Nijhof (1967).

The systems cited above were all of the "complex" type (as defined by Czerkawski (1976)), and may be unjustifiably so, for some types of research.

In many instances a simple system, such as that of Czerkawski (1969), may offer a better compromise. Some criteria for the adoption of different approaches have been set out by Czerkawski (1976) and particular stress was placed on the conflict between experimental replication and technological complexity. Whilst the use of steady-state working may reduce the need for replication, the employment of several "culture units" does allow higher work rates. Experience in the present work suggests that at least 3 fermenters are required if useful programmes of work are to be completed within a reasonable period. A further disadvantage of complex systems is that they may well hinder rather than assist the worker by demanding considerable care and attention. The observation in the review of Czerkawski (1976) that more than half of the systems cited were never used again by their authors, is particularly poignant in this respect.

Nevertheless the complex type of apparatus is becoming more important and the work of Issacson et al. (1975) on energy relationships is a good example of the worthwhile application of this experimental approach. It is also apparent that complex in vitro systems are developing along two distinct paths typified by those of Slyter (1975), Issacson et al, (1975) and that described in this thesis on the one hand, and

those of Aafjes and Nijhof (1967), Weller and Pilgrim (1974) and Thomson (1976) on the other. The essential difference between these two approaches is that whilst the former involves a highly controlled culture system working at a steady-state with a homogeneous culture, the latter is of the periodic-feed type with the bulk substrate retained in a plastic mesh capsule. The periodic feed, or cyclical, systems are likely to give greater accuracy in simulating the natural rumen and thus might be considered more suitable for "practical" studies of a nutritional nature.

The most controversial issue concerning all complex in vitro rumen systems is that of the provision of dialysis for the removal of fermentation products. This facility was considered optional by Czerkawski (1976) who, surprisingly, considered redox control to be obligatory. The evidence presented by Abe and Kumeno (1973) suggests that if low turnover rates (similar to those in vivo) are to be maintained, the removal of fermentation products by dialysis is essential. Other evidence in favour of permeability has already been presented in the literature review. The decision whether to opt for a permeable rather than impermeable system is a finely balanced one. Where actual comparisons have been made there would appear to be an advantage to the permeable type.

In vitro rumen technology has reached a stage where major innovations are unlikely. Present technology in this area is generally adequate to provide the cultural conditions required for studying rumen metabolism. Future development is likely to concentrate on refinement of existing systems.

IN VITRO - IN VIVO RELATIONSHIPS.

It has already been stated that the continuous (as distinct from cyclical) in vitro rumen cannot offer exact simulation of in vivo characteristics since the substrate-limited nature of its culture restricts substrate and product concentrations to a narrow range of values which only occur periodically in vivo. The criteria of validity of in vitro rumens set out by Warner (1956) (page 68) are thus not strictly applicable to continuous systems although it might be expected that some of these criteria, for example that concerned with overall rates of digestion, may apply. Results comparable with those in vivo have been claimed for some continuous systems. Abe and Kumeno (1973) found that at low turnover rates (0.5/d), fermentation patterns and numbers of entodiniomorph protozoa were comparable with those in vivo but only with a permeable system. Stewart, Warner and Seely (1961) and Slyter and Putman (1967) found it necessary to employ turnover rates of at least 1.5/d with impermeable systems to maintain authentic cultural characteristics. These observations are also consistent with those of Issacson et al. (1975) who found that low turnover rates led to decreased proportions of propionate. Abe and Kumeno (1973) considered this effect to be due to the removal of end products by the dilution resulting from increased

turnover, but this concept conflicts with continuous-culture theory. In a continuous culture, microbial growth rate must match increasing dilution rate or wash-out will occur. The concentration of the growth-limiting substrate, and hence metabolites, remains constant. The results of Issacson et al. indicate that as dilution rate increases, the efficiency of utilisation of available energy improves. This enhanced utilisation of energy results in the net yield of bacterial cells rising with dilution rate; an observation also in conflict with continuous-culture theory. Yield is a function of the chemical nature of the medium and is approximately constant (Powell 1965). The theory of continuous-culture is based, however, on the exponential phase of growth of a pure culture and may not fully apply to mixed cultures such as the in vitro rumen. The mean generation time of typical rumen organisms can be as low as 3.3 (h^{-1}) (Hobson 1964), suggesting that at low dilution rates considerable "internal turnover" may take place. Since the constant turnover of microbial catabolic and anabolic products will inevitably consume energy it is perhaps reasonable that increasing dilution rate results in greater efficiency of energy utilisation by the microflora. This may also explain the reduced proportions of propionate at low turnover rates since propionic acid production is associated

with the uptake of metabolic hydrogen and hence, energy conservation.

The turnover rates of cultures in the present work, whilst higher than the lowest values cited above, were none-the-less low, the mean value being $0.97 \pm 0.25\ d^{-1}$. Proportions of acetate and butyrate were high (means, 67.1 ± 5 and 11.0 ± 2.5) whilst the proportion of propionate was generally low (mean 14.7 ± 3.5). It is probable, in view of the results of Abe and Kumeno (1973) that the proportion of propionate would have been even lower in the absence of a dialysis system.

The present results allow direct comparison of microbial counts on the three selective media, between in vivo and in vitro samples. Considering together all of the eleven silages in Groups I, II and III, counts on starch and gelatin media gave high positive correlations between in vivo and in vitro (Table 45). These relationships are consistent with the effects of silage composition on ruminal microbial counts both in vivo and in vitro. Counts of cellulolytic organisms, when compared in the same way, showed no correlation, an observation which might be expected in view of the particular effects of continuous in vitro culture on these organisms (cf pages 158-161).

Table 45. Values of r for in vivo vs in vitro microbial counts and NH_3-N concentration.

	all silages	silage groups I	II	III
starch medium	0.63 (P 0.05)	0.83	0.95	-0.06
gelatin medium	0.81 (P 0.01)	0.96	0.92	0.49
cellulose medium	0.001 (N.S.)	-0.98	0.84	0.69
NH_3-N	0.482 (N.S.)	0.92	0.52	0.66

Considering the silage groups individually, the clearest relationship arose with Groups I and II, the most consistent associations occurring with counts on gelatin medium. The number of values considered for any of these correlation coefficients are low, particularly with individual silage groups, and it would be imprudent to use these figures as the basis for more extensive discussion of in vivo/in vitro relationships or for predictive equations.

Direct comparisons of the microflora were made between samples from a steer and a continuous culture inoculated with rumen contents from the same animal, by Slyter and Putman (1967). The steer and the fermenter were "fed" the same diet and after 14d 51% of bacterial isolates were identified as belonging to known genera of rumen bacteria. The same bacterial groups were predominant in the steer and the fermenter. These workers, in common with Abe and Kumeno (1973), found that protozoa were present, in reduced numbers, after prolonged culture periods in vitro.

The actual counts of viable organisms in the present experiments, both in vivo and in vitro, were lower than many reported values. Typical published counts of "total" viable bacteria (10^8 - 10^{10}) could not be expected on "selective" media, but values as

high as 10^8 have been quoted for cellulolytic species alone (Hungate 1966). Counts of 10^3 to 10^6 cellulolytic organisms per ml were made by Mann and Ørskov (1975) for a barley diet and 10^7 to $2x10^8$ cellulolytic organisms per ml were counted by Grubb and Dehority (1975) for a variety of diets including high roughage and all concentrate. The cellulolytic counts reported here were in the ranges 1.3×10^5 to 1.5×10^6 in vivo and 1.3×10^5 to 60×10^5 in vitro. These counts were somewhat lower than those quoted above. However no definitive data for viable counts on silage diets have been published and valid comparisons are precluded.

Ruminal ammonia concentrations in the Rumenstat cultures were comparable with the 2 h post feeding values in vivo (pages 95 and 160). High positive coefficients of correlation between in vitro and in vivo values were found when individual groups of silages were considered but the value of r for all silages was low (Table 45, p 204 The effects of formaldehyde treatment in Groups I and III, giving extremely low values in vitro, brought about this discrepancy.

Despite misgivings about the validity of comparisons between Rumenstat cultures and the in vivo rumen, it is apparent that the continuous in vitro cultures reflected the response of the natural rumen to different silage diets. The problems associated with certain dietary characteristics, and discussed

elsewhere (page 156 et seq.) suggest a need for caution in the use of this in vitro technique as well as a clear requirement for a wider range of experimental data to improve knowledge of these cultures and their relationship to the natural rumen.

ADDITIVE TREATMENT OF SILAGES.

The use of additives is only one of several management options available in practical silage making, and must be considered along with harvesting systems and the possible use of wilting in choosing a silage making system in practice.

The influence of different harvesting systems on fermentation and intake is less than that of wilting or the use of additives, (Hastings 1976). Chop length may influence intake through its effect on retention time and conservation (Deswysen and Vanbelle 1976).

Wilting restricts fermentation, reduces the risk of clostridial spoilage and enhances DM intake. This improvement in intake is attributable, mainly to restriction of fermentation, and the effect of dryness per se. appears to be negligible (McDonald 1975). Fermentation may also be restricted by additives and similar intake responses achieved without the need for wilting (Wilkins 1975).

Where wilting is not possible, the application of additives is desirable particularly when conditions are antagonistic to the achievement of satisfactory preservation, such as the use of regrowth material heavily fertilised with nitrogen or heavily buffered leguminous crops.

It is known that additive-treated silages are more prone to aerobic deterioration, following opening of the silo for feeding out, than lactate or wilted silages (Mann 1975, Woolford 1973). This problem can be largely overcome by minimising ingress of air, for example by the use of block cutters (Hastings 1976) and the removal of sufficient silage for immediate use only.

Nevertheless, the desirability of an additive which can remain effective throughout the ensiling period and limit aerobic deterioration on opening is clear. It is highly probable that a compound with sustained antimicrobial effect would be detrimental to rumen function. Aerobic deterioration is a complex and, at present, ill-understood subject. The nature of the attacking microflora and subsequent microbial succession depends on the composition of the silage as well as the conditions of exposure (Woolford 1976). The search for a suitable pre-ensiling additive, effective against all forms of aerobic deterioration, may be protracted.

According to Wilkinson, Wilson and Barry (1976) intake and nitrogen utilisation are low on diets of untreated silage because of protein breakdown in the silo. More than half the consumed nitrogen in such silages may be N.P.N. and readily available energy (WSC)

is fermented in the silo. Treatment with formaldehyde has been shown to increase intake and nitrogen utilisation (Barry, Fennessy and Duncan 1973; Wilkins et al. 1975; Waldo 1975; Valentine and Radcliffe 1975 and Wilkinson et al. 1976). The in vivo experiments described here showed that restriction of fermentation in the silo by formaldehyde resulted in silages with low concentrations of organic acids and protein degradation products. The formaldehyde-treated silages were associated with enhanced intake and none of the treatment levels were sufficiently high to bring about the intake-depressing effect of free formaldehyde. It has been suggested (Ferguson 1975) that formaldehyde should be used to protect dietary protein against ruminal breakdown. The protection arises from the chemical bonding of formaldehyde to various groups of specific amino acids (Van Dooren 1972) resulting in methylene cross-linkages between protein chains. These bonds are stable and resist enzymic attack in the rumen. In the present work the protection of protein in this way was confirmed, both in vivo and in vitro, by low ruminal concentrations of NH_3-N and depressed counts of proteolytic organisms.

The bonds responsible for protecting protein in the rumen are apparently hydrolysed in the acid pepsin conditions of the abomasum (Ferguson, Hemsley

and Reis 1967), resulting in an increased amino acid supply to the animal (Faichney 1974). It is remarkable that the highly adaptive microflora of the rumen are unable to disrupt bonds which are readily cleaved by the fixed and unalterable enzyme systems of the abomasum, particularly since the relatively small volume of this organ must dictate a short residence time for the ingesta. Excessive rates of application of formaldehyde may result in either nitrogen starvation of the rumen microflora or direct inhibition in the rumen by residual free formaldehyde. Barry (1976) considered that, taking into account field and ensiling losses, the optimum rate of formaldehyde application would be 30 - 50 g/kg degradable true protein. This figure was arrived at after consideration of a range of experimental results from several authors, with particular reference to the growth responses of animals offered formaldehyde-treated diets. Precise figures for the capacity of forage protein to bind with formaldehyde were not given and definitive data on this matter are urgently required. It is likely that complete binding of dietary protein is undesirable and, apart for the obvious need to avoid the occurrence of free formaldehyde in the rumen, the nitrogen requirements of cellulolytic organisms of the rumen must be taken into account. The influence of formaldehyde treatment on nitrogen availability was particularly

evident with the in vitro cultures where TVFA production was depressed under conditions of nitrogen limitation. These effects which were in part due to the substrate-limited nature of the in vitro culture, were not shown in vivo.

The organic acid additives investigated in these experiments resulted, like formaldehyde, in compositional changes to treated silages. The infusion experiments confirmed that concentrations of organic acids likely to arise in the rumen as a consequence of silage treatment would not significantly effect ruminal metabolism.

Formic acid is widely used as an additive and has been shown to be effective in controlling fermentation and improving intake (Waldo et al. 1969; Castle and Watson 1970). The results of in vivo work reported here confirm this and effects on rumen characteristics were as expected in view of changes in silage composition. This was confirmed by the similar ruminal patterns which were obtained in vitro.

In vitro infusion data indicated that even at high rates of infusion, corresponding to concentrations several times greater than would arise with additive treated silages, formic acid had little effect. The higher acids, acetic and propionic, were more inhibitory but even these were not significantly

detrimental at practical concentrations.

On the basis of published work and the present data formic acid would appear to be particularly suitable as a silage additive. Mixtures of formic acid and formaldehyde, relying on the acid for preservation and using formaldehyde to protect the protein could be used in silage making. Such mixtures have been used (Wilkins et al. 1975 and present work) with apparent benefit on preservation quality and nutritive value. Work aimed at defining rates of application and optimal ratios of the two compounds, to suit various ensiling situations, is highly desirable.

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APPENDIX 1.

1.A CONTINUOUS CULTURE THEORY.

During the exponential phase of growth of a microroganism in batch culture, the medium becomes depleted and waste products accumulate. In continuous culture, fresh nutrients are added and waste products removed throughout the culture period.

The exponential rate of growth of a micro-organism (μ) is given by the following equation :

$$\mu = \frac{1}{X} \cdot \frac{dX}{dt} \quad \text{.....(A1.1)}$$

where X is the concentration of organisms in the culture.

From this equation it follows that the rate of increase of growth :

$$\frac{dX}{dt} = \mu X \quad \text{.....(A1.2)}$$

In a continuous-culture system where the volume of the culture in the vessel (V) is constant and the flow rate in thereby equals the flow rate out (f) the dilution rate will be :

$$D = \frac{f}{V} \quad \text{.....(A1.3)}$$

Thus the rate at which organisms are "washed out" of the vessel will be :

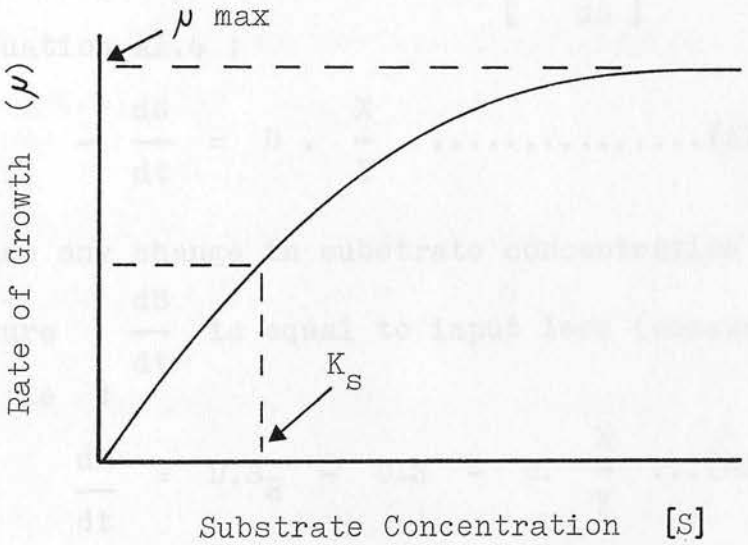
$$- \frac{dX}{dt} = DX \quad \text{..... (A1.4)}$$

The change in the number of microorganisms in the culture will thus be :

$$\mu X - DX \text{ or } X(\mu - D)$$

When a culture is at a steady-state there is no change in X (number of organisms present) therefore $\mu = D$

The relationship between μ and the concentration of substrate (S) is as follows :



μ max is the maximum rate of growth possible in the medium. K_s is a saturation constant numerically equal to the substrate concentration which will support a growth rate half the maximum $(\frac{\mu \text{ max}}{2})$. The curve above is described by the equation :

$$= \max \left[\frac{S}{K_s + S} \right] \quad \text{..... (A1.5)}$$

Since, at steady-state = D we may write

$$D = \mu \max \left[\frac{S}{K_s + S} \right] \quad \text{.....(A1.6)}$$

This equation indicates that any value of obtained in continuous culture must be less than μ max.

The value of X is unaffected by S_R, the concentration of the growth limiting substrate in the fresh medium.

Rate of substrate utilisation :

$$- \frac{dS}{dt} = - \frac{dS}{dX} \cdot \frac{dX}{dt} \quad \text{.....(A1.7)}$$

The yield (Y) is the weight of organisms produced for weight of substrate used $\left[- \frac{dX}{dS} \right]$ and applying equation A1.4 :

$$- \frac{dS}{dt} = D \cdot \frac{X}{Y} \quad \text{.....(A1.8)}$$

Since any change in substrate concentration in the culture $\frac{dS}{dt}$ is equal to input less (consumption + outflow) ie :

$$\frac{dS}{dt} = D.S_R - D.S - D. \frac{X}{Y} \quad \text{...(A1.9)}$$

At steady-state $\frac{dS}{dt} = 0$ therefore :

$$D.S_R = D.S - D. \frac{X}{Y} \quad \text{.....(A1.10)}$$

or $$X = Y (S_R - S) \quad \text{.....(A1.11)}$$

Equations A1.4 and A1.11 allow the prediction of steady-state concentrations of organisms and substrate for any values of D and S_R provided values of μ max, K_s and Y are known.

1.B CALCULATION OF VFA PRODUCTION RATES IN VITRO.

The calculations of VFA production rates for in vitro systems cited, for which actual figures are not given, are based on the following assumptions :

1) The VFA is produced in the ratio C_2 : C_3 : C_4; 65 : 20 : 15 (Wolin 1960)
2) That all VFA is derived from cellulose hydrolysis.

Thus :

$$(\text{GLYCOSYL})_n + (H_2O)_n \longrightarrow n \text{ GLUCOSE}$$

$$\text{MW} \quad (162.15)_n + (18.1)_n \longrightarrow (180.16)_n$$

$$\text{So glucose available} = \text{cellulose digested} \times \frac{180.16}{162.15}$$

$$= \text{cellulose digested} \times 1.111$$

Table A1.1 shows the derivation of factors for the calculation of VFA concentrations, from glucose consumed, on the basis of assumptions 1 and 2 above.

Calculations for results cited :

a) Warner (1956). Figures suggest that 80 mg of cellulose was digested in 8 h in a 50 ml culture. This corresponds to 0.02222 g of glucose/100 ml/h.

The VFA production rate estimate is thus :

C_2	5.612 x 0.0222	=	0.125
C_3	0.043 x 0.0222	=	0.043
C_4	0.027 x 0.0222	=	0.027
	TVFA	=	0.195 mM/100 ml/h

b) Harbers and Tillman (1962). The range of values for rate of cellulose digestion, based on culture volume and time were 0.0061 to 0.02289 g/100/h (glucose equivalent). If the calculation is based on overall flow rate these values become 0.020 to 0.1842 g/100/h (glucose equivalent).

The VFA production rate estimate is thus :

	culture volume basis		medium flow basis	
	0.0061 -	0.02289	0.020 -	0.1842
C_2	0.0343	0.1285	0.112	1.0337
C_3	0.0118	0.0442	0.0386	0.3556
C_4	0.0074	0.0275	0.0241	0.2216
TVFA = (mM/100 ml/h)	0.0535	0.20	0.1747	1.6108

Table A1.1 Derivation of factors for conversion of glucose consumed to VFA produced in a 65 : 20 : 15 fermentation ratio.

VFA	%	LCD	MW	VFA LCD X MW	glucose consumed LCDxMW	proportion of glucose consumed	VFA : glucose ratio	g individual VFA produced from 1g glucose	mM VFA produced from 1g glucose
acetic	65	13	60.05	780.65	1171.0	0.565	0.667	0.377	5.612
propionic	20	4	74.08	296.32	360.3	0.174	0.822	0.143	1.930
butyric	15	3	88.10	264.3	540.5	0.261	0.489	0.106	1.203

c) Davey, Cheeseman and Briggs (1960). The TVFA production rate quoted is derived from graphical data, not cellulose digestion figures. The culture volume was 850 ml and calculated on that basis the production rate is 0.1838 mM/100 ml/h. Calculated in the culture flow rate of 500 ml/d, the TVFA production rate was 0.2918 mM/100 ml/h.

1.C ADDITIVE INFUSION EXPERIMENTS.

a) Calculations of amounts of additives injected : The injections contained the amount of additive compound required to change the concentration in the culture from that of one experimental period to that of the next.

eg Formic acid, period 2 = 50 mM/l
and, period 3 = 100 mM/l
difference = 50 mM/l
on 0.0023 g/ml

$\therefore$ injection size = 0.0023 x 450
= 1.035 g formic acid (MW = 46)
= 1.53 g sodium formate (MW = 68)

The amounts calculated for the injections are given in Table A1.2.

b) Calculation of infused concentrations : In order to maintain a nominal C_v of infused additive, the calculation was based on an apparent concentration twice the nominal value (see page 168). Thus, for example,

Table A1.2a. Details of additive infusion experiments.

period		1	2	3	4	5	6
nominal concentration (mM/l) of infused additive		25	50	100	200	400	800
formic acid	concentration (g/ml)	0.0012	0.0023	0.0046	0.0092	0.0184	0.0368
	injection (Na salt) (g)	0.76	0.76	1.53	3.06	6.12	12.23
	infusate conc. (g/ml)	0.035	0.069	0.138	0.276	0.552	1.104
acetic acid	concentration (g/ml)	0.0015	0.003	0.006	0.012	0.024	
	injection (Na salt) (g)	1.58	1.58	3.06	6.12	12.24	
	infusate (g/ml)	0.045	0.09	0.18	0.36	0.72	

Table A1.2b. Details of additive infusion experiments.

period		1	2	3	4	5
nominal concentration (mM/l)		25	50	100	200	400
propionic acid	concentration (g/ml)	0.0019	0.0037	0.0074	0.0148	0.0296
	injection (Na salt) (g)	1.08	1.08	2.17	4.32	8.65
	infusate conc. (g/ml)	0.056	0.111	0.222	0.444	0.889
formal-dehyde	concentration (g/ml)	0.0008	0.0015	0.003	0.006	
	injection (g)	0.34	0.34	0.68	1.35	
	infusate (g/ml)	0.023	0.045	0.90	0.180	

the 50 mM/l C_v periods were maintained at that concentration by infusate concentration calculated for a value of 100 mM/l C_v.

Considering equation (8.4) we may derive :

$$C_{add} = \frac{C_v \times F}{F_{add}}$$

and since F_{add} = 1(ml/h) and F = 15 (ml/h)

$$C_{add} = C_v \times 15$$

So that, for example, the 50 mM/l period with formic acid required a solution to maintain C_v at 100 mM/l (apparent) :

$$C_{add} = 0.0046 \text{ g/ml} \times 15$$
$$= 0.069 \text{ g/ml}$$

The concentrations calculated for infusates are given in Table A1.2.

1.D AUTOMATIC DISPENSER.

The automatic dispenser was derived to meet two specific requirements for distribution of rumen-organism media: The maintenance of anaerobiosis and the constant resuspension of particulate components.

The equipment employed a peristaltic pump (with two running speeds) delivering media through gas impermeable butyl rubber tubing. The pump was in a

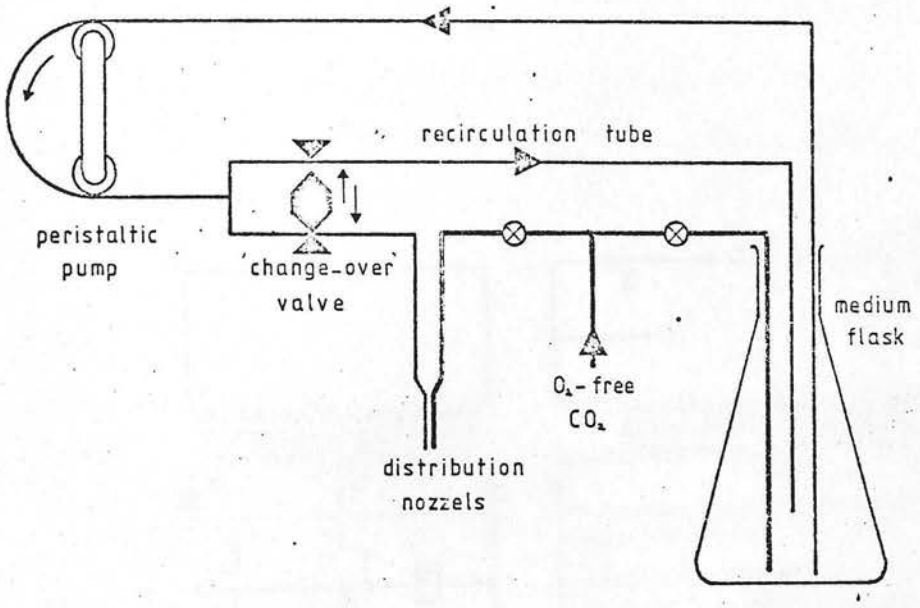


Fig. A.1.1 Recirculating circuit of automatic dispenser.

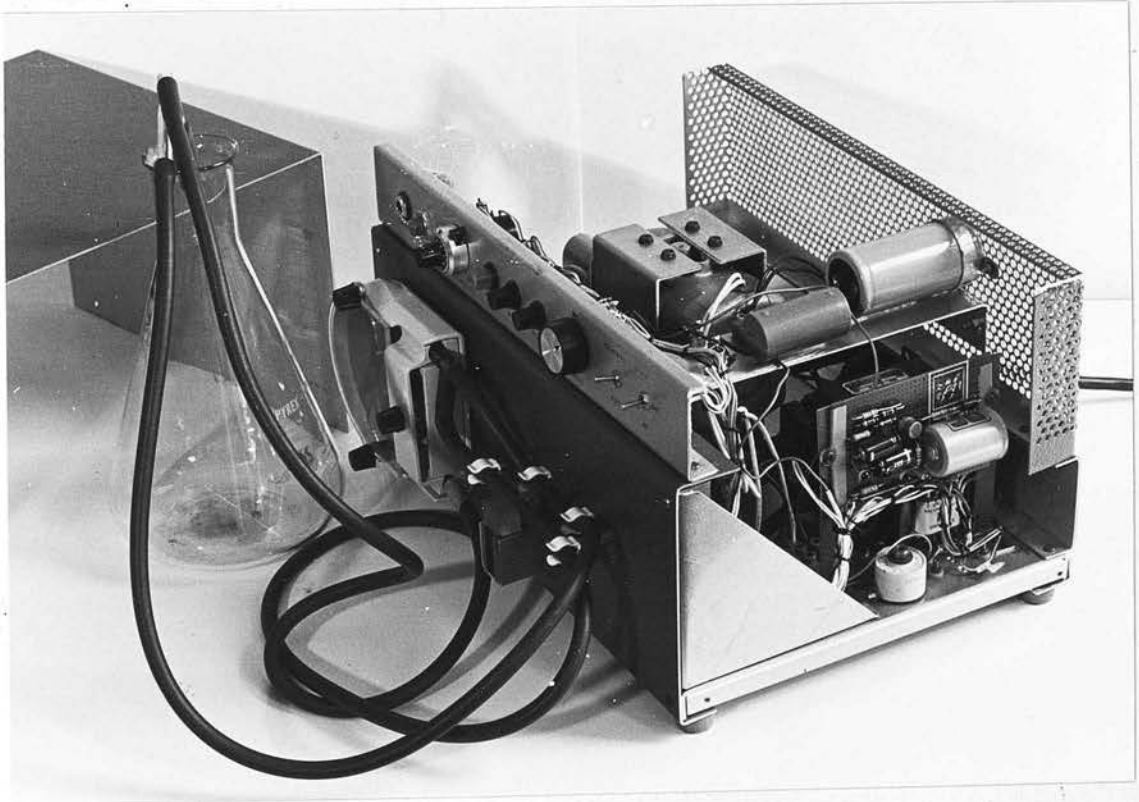


Fig. A.1.2 Automatic dispenser (cover removed).

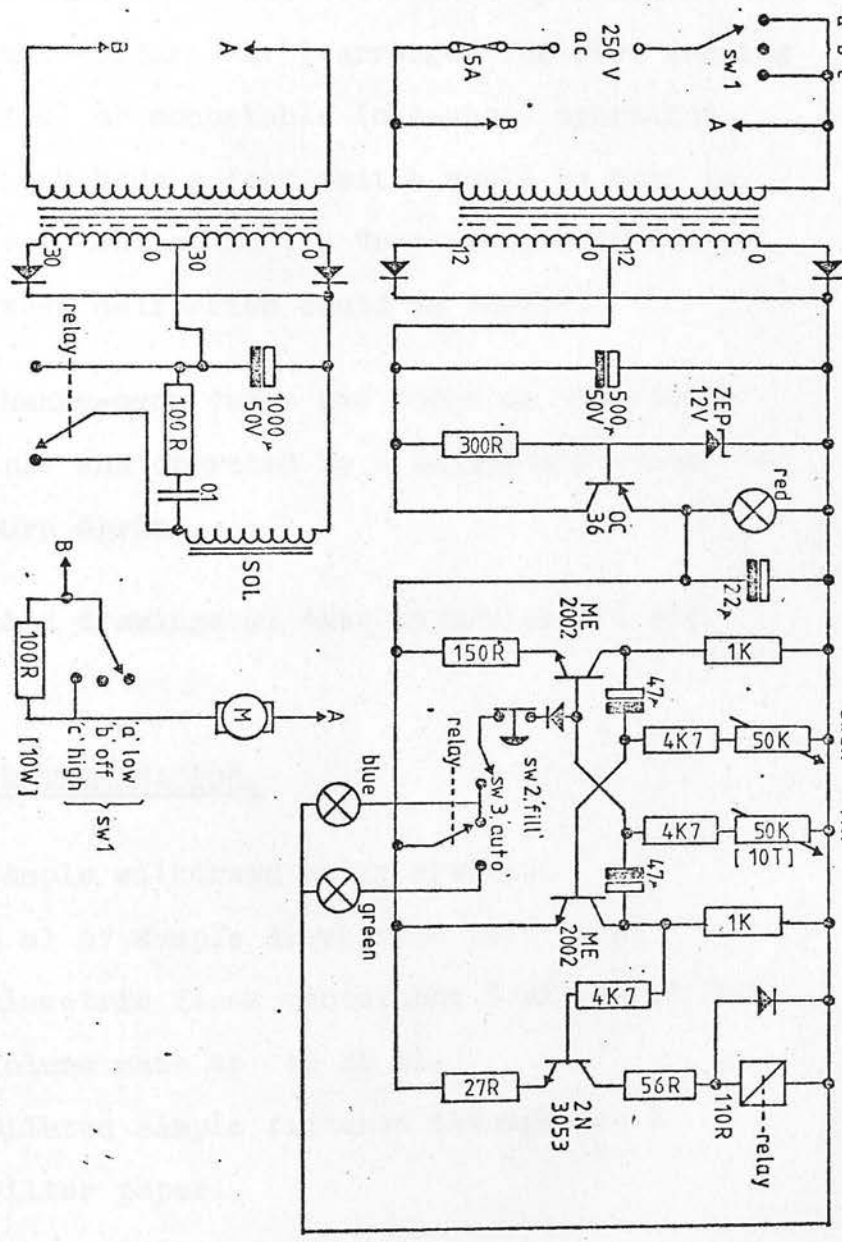


Fig. A.1.3 Circuit of automatic dispenser.

recirculating circuit (Figure A1.1), which maintained suspensions, and the media were distributed by a change-over valve arrangement which temporarily redirected pump output to a dispensing nozzle, for a fixed time interval. The length of the time interval (ie the volume delivered) was determined by a multi-vibrator circuit (Figure A1.3) arranged for free running (repeated action) or monostable (one-shot) operation. In the "one-shot" mode a foot switch could be used to initiate delivery whilst in the "repeat-action" case, the dwell between deliveries could be varied.

The change-over valve was based on the "tube-pinch" principle and operated by a heavy-duty solernoid, against a return spring.

Detailed drawings of this apparatus are not included.

1.E NH_3-N DETERMINATION.

1. Sample withdrawn using syringe.
2. 5 ml of sample discharged into 25 ml volumetric flask containing 5 ml of 0.1 NHCl.
3. Volume made up to 25 ml.
4. Diluted sample filtered through No. 1 filter paper.
5. Conway units prepared :
   a) outer chamber 1 ml saturated potassium carbonate.

b) centre chamber 1 ml boric acid/ indicator solution (5g boric acid, 200 ml ethanol, 700 ml water, 10 ml indicator adjusted to end point with NaOH).

c) lids smeared with soluble fixative (30 ml water, 20 g gum arabic (acacia-B.D.H.), 10 ml glygcerol, 10 ml saturated potassium carbonate).

6. 1 ml of filtrate pipetted into outer chamber, avoiding saturated potassium carbonate. (Blank and standard)

7. Lid of Conway unit carefully fitted and outer chamber materials mixed.

8. Diffusion allowed at least 4 h.

9. Centre chamber titrated 0.01 NHCl.

1.F GLC TECHNIQUES.

Details of GLC determination of ruminal VFA are given in Table A1.3.

Response was linear over the concentration range of each acid. Repeatibility with each instrument and reproducibility between the columns and instruments was within 0.01 for the major acids (C_2, C_3 and C_4). A solution containing the VFAs being determined, in known concentration, was subjected to the same deproteinisation and centrifugation as the samples and used as standard. The higher VFAs were present in small amounts, and to increase the accuracy of their measurement, amplifier attenuation was decreased. In some cases attenuations lower than those given in the table below were used.

Table A1.3 Details of GLC determination of ruminal VFA.

instrument :	Perkin-Elmer F11	Pye 104
size of sample	2 l	0.7 l
detector	flame ionisation	flame ionisation
column material	glass	glass
column size	1.8m x 6mm OD	1.5m x 4mm OD
stationary phase	FFAP(1)	neopentyl glycol adipate
support	Chromosorb G (80 - 100 mesh)	Diatomite C (100 - 120 mesh)
amount of stationary phase	5%	20%
carrier gas	argon	argon
c/gas pressure (bar)	1.52	1.21
H_2 pressure (bar)	0.97	0.69
air pressure (bar)	1.72	1.72
column temperature (^oC)	175	-
recorder	Kent Mark 3	Leeds & Northrup Speedomax W
chart speed	380 mm/h	254 mm/h
attenuation(2)	C_2, 5 x 10^2 C_3+C_4, 2 x 10^2 C_5+C_6, 50 x 1	C_2, 5 x 10^2 C_3+C_4, 2 x 10^2 C_5+C_6, 50 x 1

(1) Manufactured by Perkin-Elmer

(2) C_2 - acetic acid; C_3 - propionic acid;
C_4 - butyric acid; C_5 - valeric acid;
C_6 - hexanoic acid.

1.G TUBE ROLLER.

The tube roller comprised of a motor assembly and a trough. A rubber roller driven by a synchronous motor, (250 rpm) and arranged on a counter-balanced pivot, rotated the tube. The tube lay astride two pairs of "Derlin" (P.T.F.E. - loaded nylon) rods which acted as bearings. Careful adjustment of the levels of cooling water in the trough allowed the tube to spin freely and brought about rapid and regular gelling. Waste cooling water drained into the water bath below the roller (Figures A1.4 and A1.5).

Plastic-cated aluminium sheet was used for construction of the roller assembly and the water trough was sealed with silicone rubber compound.

In use the closed (glass) end of the inoculated tube was placed under the roller and the tube lowered along the trough, lifting the roller assembly against the counter balance such that driving force was applied to the tube wall.

To ensure that the stoppered end of the tube did not "run out" against the trough end plate, the axis of the roller was placed at a slight angle to the tube centre line to provide a "screw" action which held the glass end of the tube against the rear of the trough.

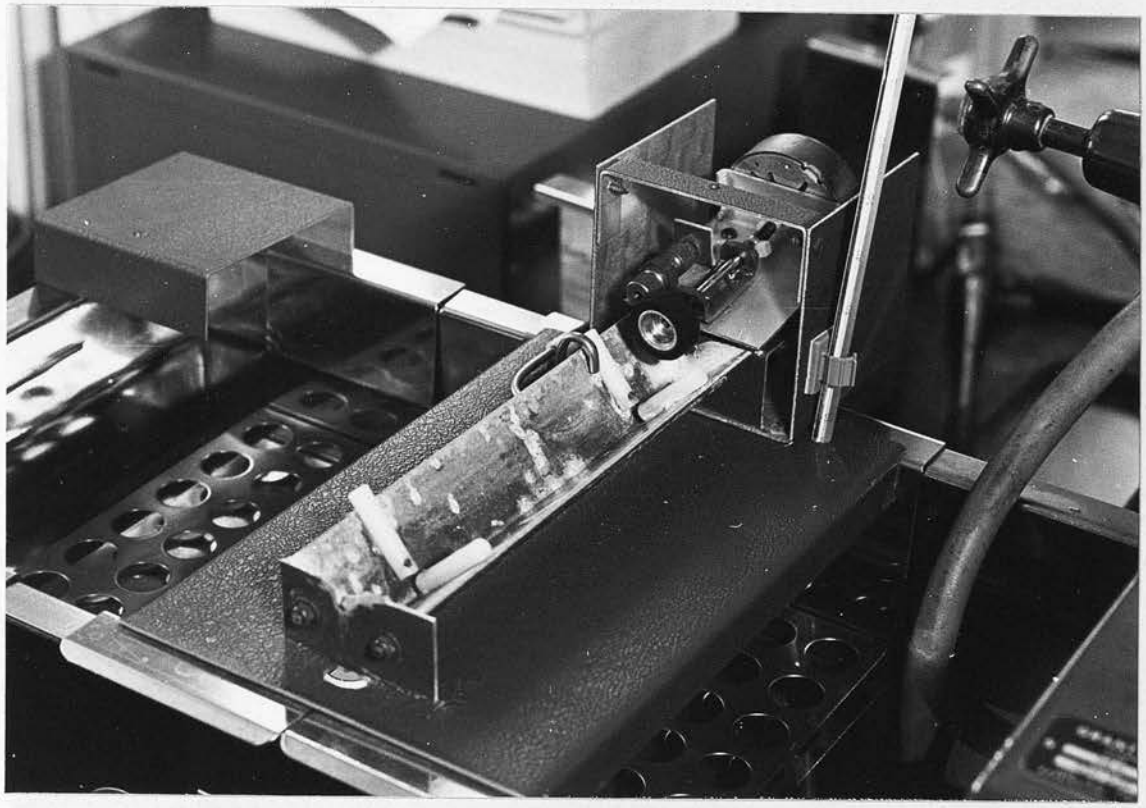


Fig. A.1.4 Tube roller.

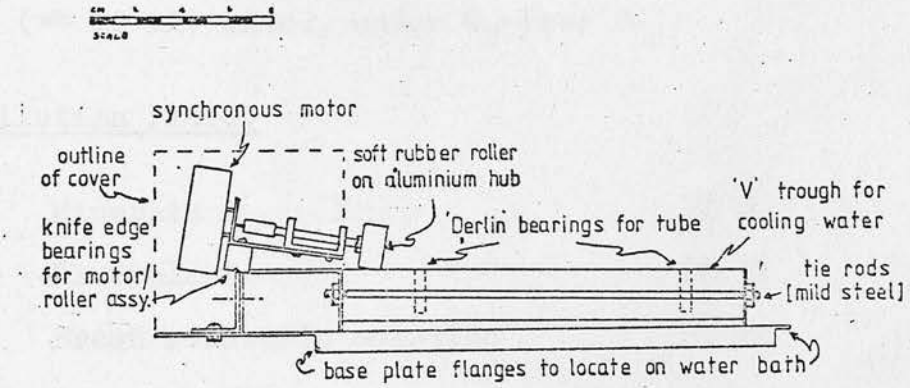


Fig. A.1.5 Diagram of tube roller.

1.H. MEDIA FOR VIABLE COUNTS.

1. Stock solutions.

a) Minerals A :	KH_2PO_4	1.5 g
	$(NH_4)_2SO_4$	3.0 g
	NaCl	3.0 g
	$MgSO_4$	0.3 g
	$CaCl_2$	0.3 g
b) Minerals B :	K_2HPO_4	3.0 g

c) Clarified rumen fluid : Rumen contents strained through 2 layers of muslin and adjusted to pH 7.0. Centrifuged at 60,000 G for 10 mins then filter-sterilised.

d) Resazurin solution : 1 tablet (BDH) in 100 ml water.

e) Bicarbonate/Dithionite solution : 500 ml of 10% $NaHCO_3$ autoclaved in screw-cap bottle then 0.3 g of sodium dithionite dissoved in a minimum of boiling water ($\simeq$ 10 ml) added, under O_2-free CO_2.

2. Dilution Fluid.

Minerals A	75 ml
Minerals B	75 ml
Fresh resazurin solution	50 ml
Cysteine HCl	0.25 g
Tryptose	3.0 g
water to	480 ml

Boiled under CO_2, 20 ml of bicarbonate

dithionite solution added and, when completely reduced, dispensed under O_2-free CO_2 into narrow-neck vials. Sterilised 121°C/ 15 mins.

3. Rumen-fluid media.

A basal medium was made us as follows (amounts are given for 600 ml = 3 x 200 ml)

Minerals A	35 ml	
Minerals B	35 ml	
Resazurin	20 ml	3 flasks
Cysteine HCl	0.1 g	
Agar	3.5 g	

Additional ingredients were as follows :

Flask 1 (starch medium)

Starch	1 g
Tryptose	0.8 g

Flask 2 (gelatin medium)

Gelatin	2 g
Glucose	0.5 g

Flask 3 (cellulose medium)

Cellulose powder (CC 41)	1 g
Tryptose	0.8 g

Contents of each flask completely dissolved in water and volume adjusted to 170 ml. The media

were autoclaved at 121°C for 15 minutes. 10 ml of bicarbonate dithionite solution and 20 ml of clarified rumen fluid were added and the media saturated with O_2-free CO_2. The media were dispensed in 2.3 ml amounts, under O_2-free CO_2, by pipette or using an automatic dispenser (Appendix 1.D).

1.I SEEDING MIXTURES.

Group I and Group III silages.

Seeding mixture of Lower Lambing field.

2nd year ley.

Leda Italian Ryegrass	4
Presto Perennial Ryegrass	6
Barvestra Perennial Ryegrass	6
Barlatra Perennial Ryegrass	6
S 23 Perennial Ryegrass	6
Altaswede Red Clover	3
N.2. White Clover	2
	33 lbs/acre

Group II silage.

Jean Lowrie Field :
EF 486 Dasas Italian Ryegrass - 30 lbs/acre

Howgate Stackyard Field :
EF 486 Dasas Italian Ryegrass - 40 lbs/acre

S 37 silage.

Tetila Tetraploid Italian Ryegrass	10
EF 486 Italian Ryegrass	25
	35 lbs/acre

APPENDIX 2.

2.A CULTURE VESSEL.

The culture vessel cover was constructed from 1.62 mm (16 swg) stainless steel plate 154 mm x 154 mm drilled to accomodate the fittings shown in Figure A2.1. Draw-blots which secured the vessel between the magnetic stirrer (below the vessel) and the cover, were mounted through holes on centres 105 mm x 105 mm. The draw-bolts were 4.75 mm (3/16") diameter mild steel.

Rubber grommets were inserted into the openings which received the pH, Eh and reference electrodes, as well as those for the gas/sampling tube and the effluent weir. The port through which feed was delivered was extended above the cover by a 25 mm length of 16 mm borosylicate tubing (1.5 mm wall) secured by self-curing silicone rubber compound. The feed delivery tube was fitted through a rubber grommet at the top of the extension tube and reached down to the level of the cover surface. This arrangement allowed a "flow break" in the feed delivery to reduce contamination of the fresh feed, with culture.

The remaining ports were fitted directly with stainless steel tubing secured by epoxy resin adhesive and made gas-tight by a further application of silicone rubber compound.

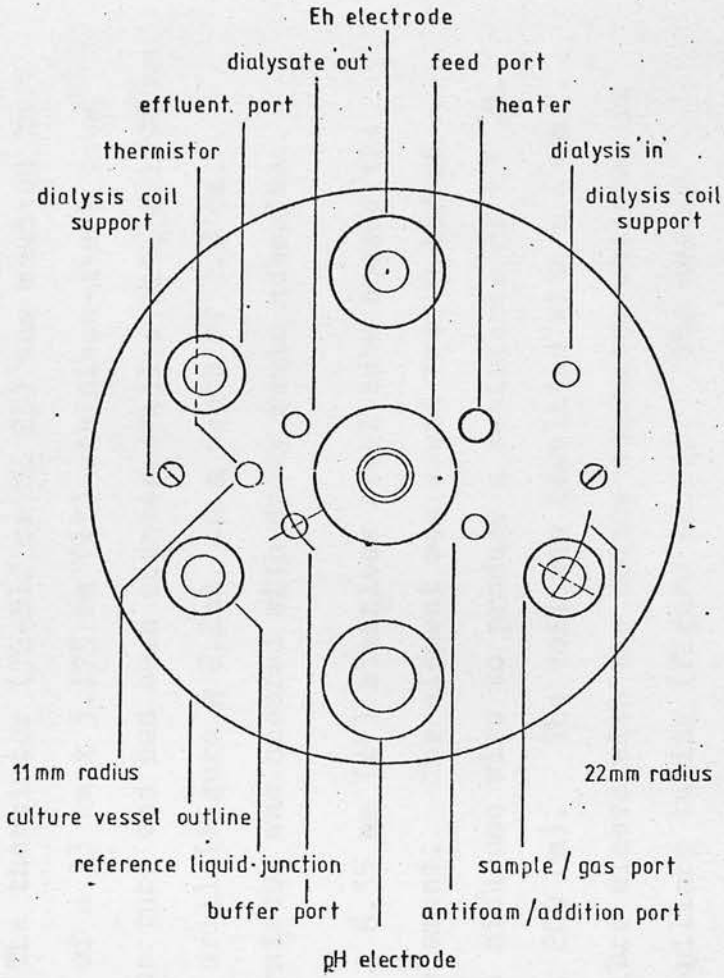


Fig. A.2.1 Culture vessel cover details.

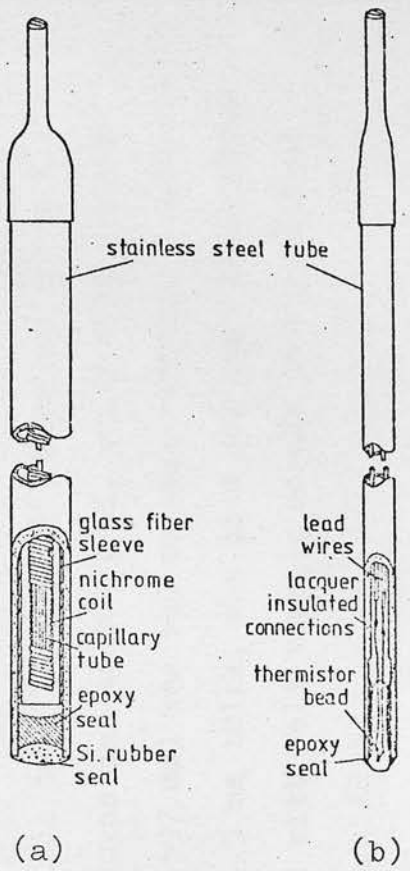


Fig. A.2.2 Culture heater (a) and thermistor probe (b).

Thermistor and Heater Probes.

The thermistor (TH-B12 or GL 23) was mounted in the end of a 13 cm x 3.175 mm ($\frac{1}{8}$") stainless-steel tube after the tube end had been counter-drilled with a 1.98 mm (5/64") drill (Figure A 2.2a), to a depth of 1.0 cm. The thermistor was secured with epoxy resin adhesive.

A 6.35 mm ($\frac{1}{4}$") stainless steel tube housed the heater element. The element was wound from 0.152 mm (38 swg) nichrome wire to produce a resistance of 100 Ω (approx. 200 cm). The coil was insulated with a 4 mm glass-fibre sleeve with the centre conductor enclosed in glass capillary tubing (Figure A2.2b). The heater element was rated at 50 W nominal and powered from a double-wound mains transformer with secondary at 50 V, centre tapped to earth.

2.B FURNACE ASSEMBLY.

The design of the furnace was based on drawings supplied by P.N. Hobson (Rowatt Research Institute).

A silica tube 17.8 mm (7") long and 32 mm ($1\frac{1}{4}$") o.d. with grooves cut at 20 T.P.I. wound with nichrome wire (22g, 0.597 mm) was the furnace element. Asbestos cement sheet, 5 mm thick, was used for the furnace body and assembled with self tapping screws on the centres shown in Figure A2.3.

An aluminium heat shield (2.032 mm, 14g),

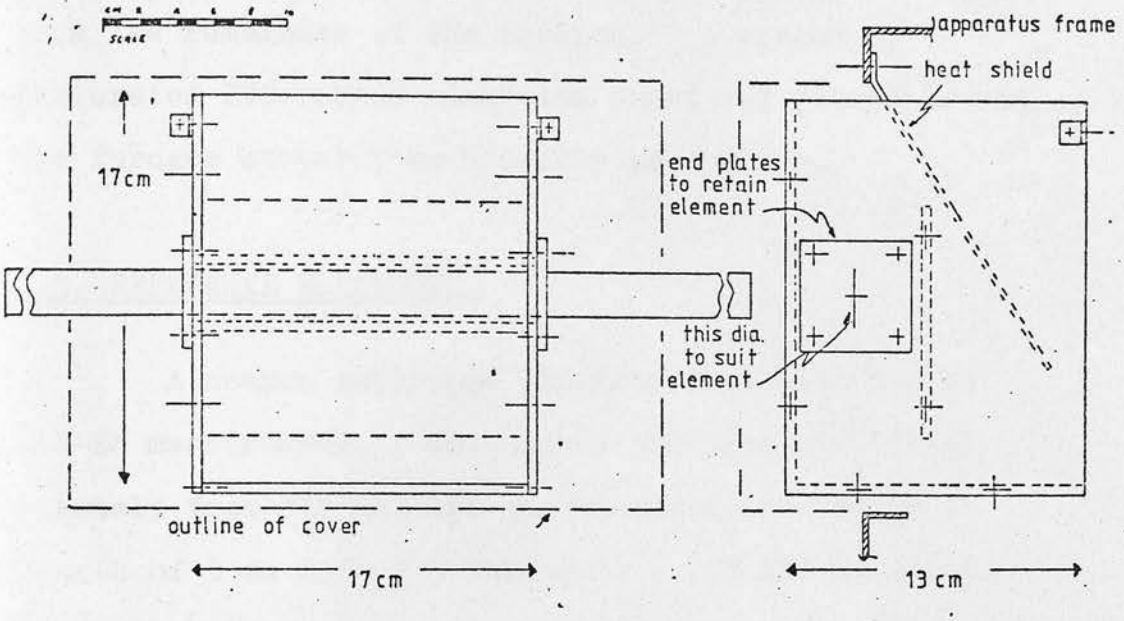


Fig. A.2.3 Diagram of furnace assembly.

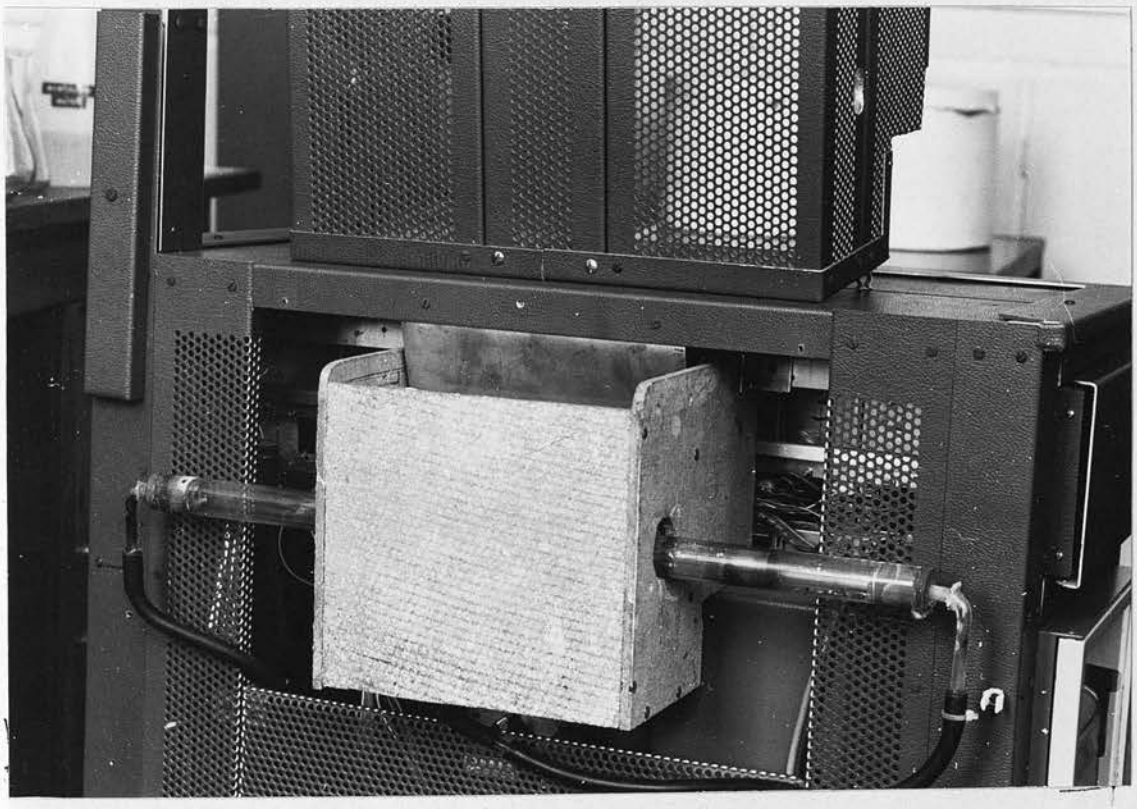


Fig. A.2.4 Furnace assembly on rear of apparatus main-frame (cover removed)

mounted on the apparatus main frame, deflected heat from the remainder of the machine. A screen of perforated PVC-coated aluminium sheet was placed around the furnace assembly as a safety precaution.

2.C REFERENCE ELECTRODE.

A common reference electrode was used for pH and Eh measurement. The liquid junction was fitted remotely from the culture vessel and connected by a length of 5 mm i.d. PVC tubing to a 200 x 5 mm glass tube which tapered to a 1.5 mm orifice. The glass tube was filled with agar/KCl to provide the liquid junction at the orifice.

The agar/KCl reference junction was made as follows :

KCl	40 g
Agar	2.5 g
Water	70 ml

Autoclave 121°/ 15 minutes, mix and draw into tapered glass tube. Gel promptly under running cold water.

2.D DIALYSIS SYSTEM.

A 30 cm length of Visking tubing (22 mm flattened width) coiled round a former of 3 mm polythene mesh provided the dialysis membrane (Figure A2.6). The tube walls were kept apart by a 2 mm

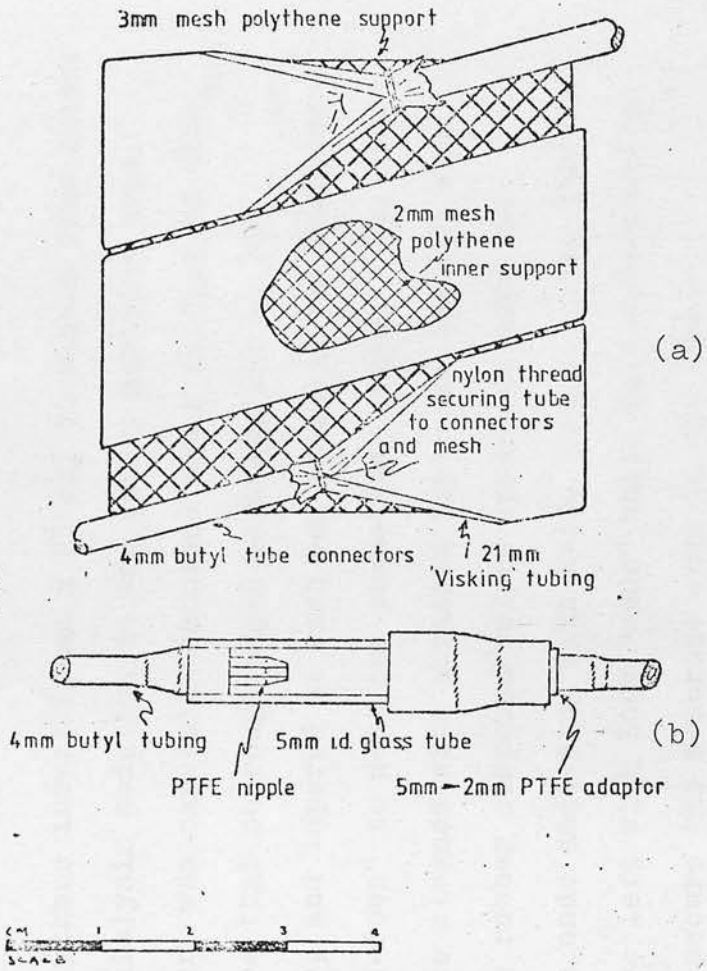


Fig. A.2.5 Diagram of dialysis coil (a) and flow breaks (b).

Fig. A.2.6 Dialysis coils; left, unused coil; right, coil after 180 h use.

mesh polythene insert 20 mm x 28 cm, to ensure free access of the dialysis solution to the entire membrane area. The insert was carefully smoothed after cutting to size, to ensure that no rough edges were applied to the membrane, and tapered at each end to allow the membrane to "fold-down" to meet the connecting tubes. The tube ends were cleaned with abrasive paper and smeared with silicone rubber compound before insertion into the membrane ends and tying with nylon thread. The thread ties were left with long "ends" which were subsequently used to secure the membrane ends to the former.

It was found necessary to exercise great care in handling the Visking tubing during assembly of a dialysis coil. Slight damage or stress to the membrane during this procedure was considered to be responsible for most of the membrane failures which occurred. Any membranes showing local "stress points" in contact with the linear mesh, particularly at the ends, were discarded and not put into service.

The flow-breaks fitted to the dialysis delivery and effluent pipes, and described in the text, are shown in Figure A2.5.

2.E MAGNETIC STIRRERS.

Adequate torque at low stirring speeds was obtained from the magnetic stirrers by adopting a belt

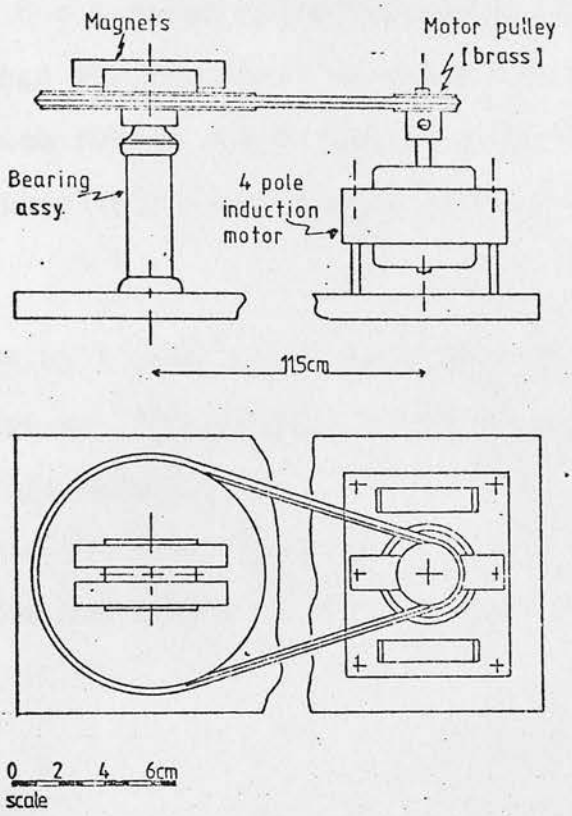


Fig. A.2.7 Diagram of magnetic stirrer.

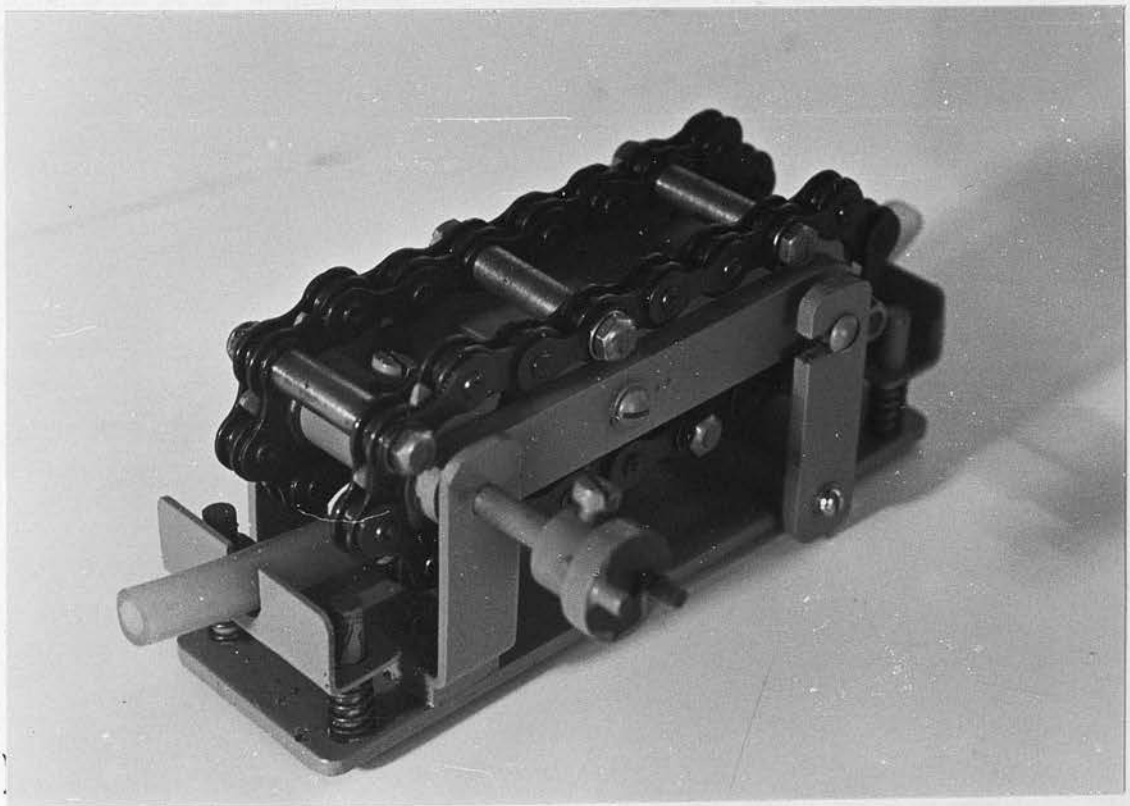


Fig. A.2.8 Linear pump.

drive to give an 8 : 1 speed ratio between motor and magnets. Bearings for the shaft carrying the magnets required to be both robust and reliable, properties readily and inexpensively available in bicycle pedal hub bearings.

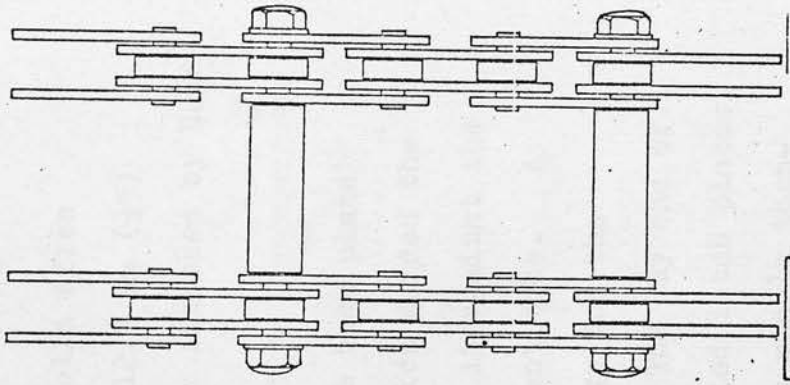
Drive was by 4 pole induction motors *via* neoprene belts (C.E.S. part no. 358 30078) running on brass driving pulleys and acrylic driven pulleys. The layout and general dimensions of these units are given in Figure A2.7. Speed controls for the stirrers are described in Appendix 2.K.

2.F LINEAR PUMP.

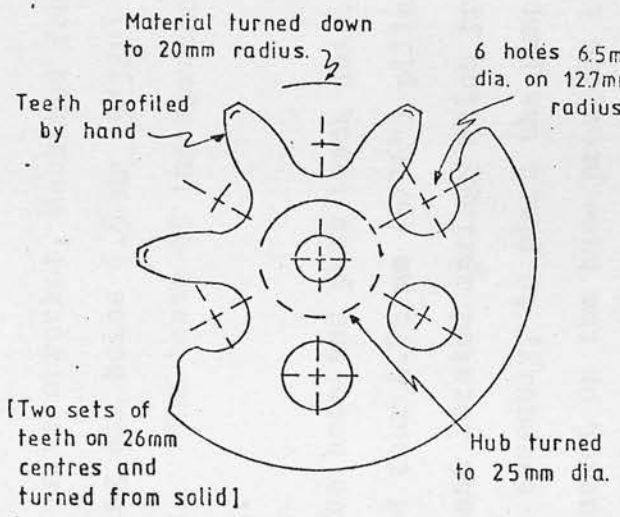
The pump comprised four parts :

1) The roller assembly consisting of two loops of 12.7 mm ($\frac{1}{2}$") pitch roller chain with every third rivet removed from each loop and replaced by an adjoining 3.175 mm ($\frac{1}{8}$") steel rod carrying a 6.35 mm ($\frac{1}{4}$") o.d. 19.05 mm ($\frac{3}{4}$") long chromium plated brass roller between the loops.

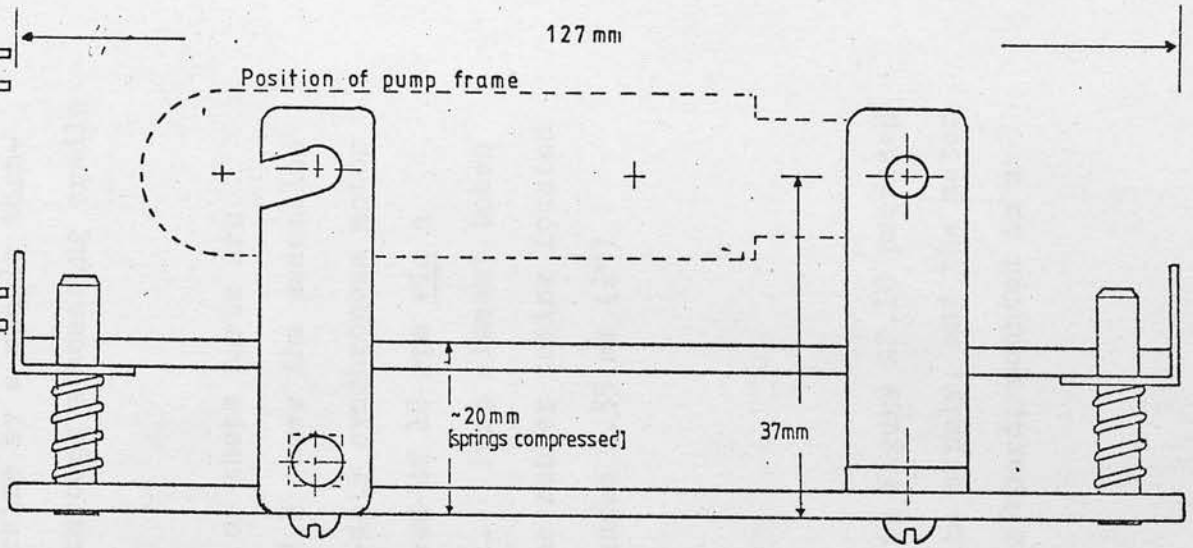
2) The pump frame comprising of two 3.175 mm ($\frac{1}{8}$") side plates profiled to guide the chain loops along the length of the roller track and round the drive sprocket. These side plates were bolted to a 6.35 mm ($\frac{1}{4}$") centre piece, of width to suit the roller assembly, and to the frame rails which carried the drive sprocket shaft.



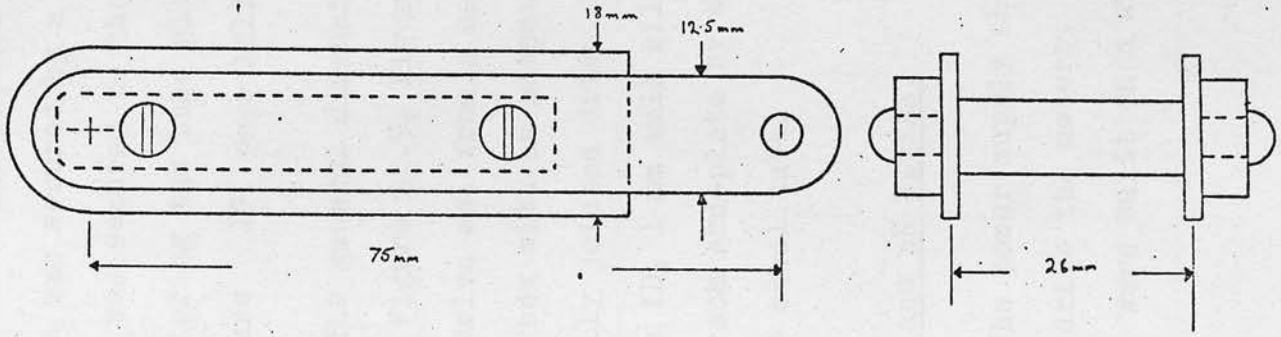
1. Roller Assy. [part]



3. Drive Sprocket



4. Base And Pump Track



2. Pump Frame

Fig. A.2.9 Diagram of components of linear pump.

3) A drive sprocket, machined from a solid after drilling six holes 6.5 mm (9/32") on a 12.7 mm (½") radius. The teeth of the sprocket were finished by hand filing.

4) The base and pump track unit. The base plate carried four 4.76 mm (3/16") pillars which guided the track and located springs which pressed it against the roller assembly, to pinch the pump element tube. A 'U' bracket on the base provided bearings for the sprocket shaft and located the frame. The lay end of the frame was secured by a pair of slotted latch plates. The pump was secured to the machine by a single turn-screw entering the base plate through a mounting cradle (See Figure 19, page 121).

The general dimensions of these parts are shown in Figure A2.9; Figure A2.8 shows the assembled pump. Drive was from a heavy-duty synchronous motor and gear box with an output speed of 10 rpm *via* a universally jointed drive shaft. Pump element tubes were 5 mm ID, 1 mm wall silicone rubber tubing located on the track end-plate by a flanged 6.35 mm (¼") stainless steel tube.

2.G Eh and pH METERS.

The power supply shown in Figure A2.10 provided ± 5 V to drive the meters. This supply, and the meter circuits, were built on a single board, mounted in a

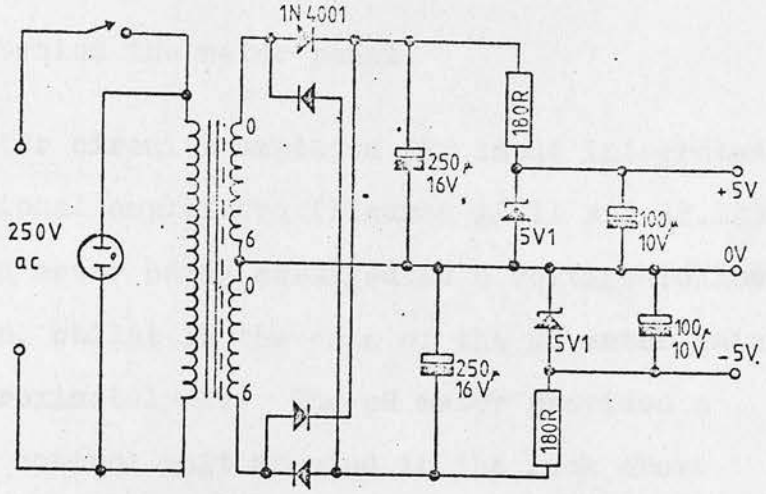


Fig. A.2.10 Power supply for pH and Eh meters.

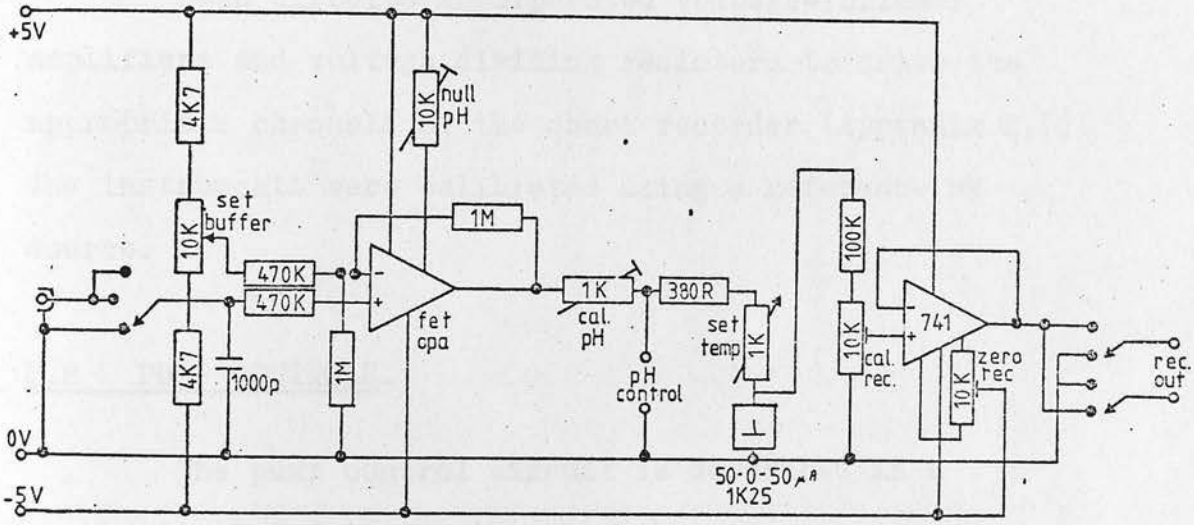


Fig. A.2.11 pH meter circuit.

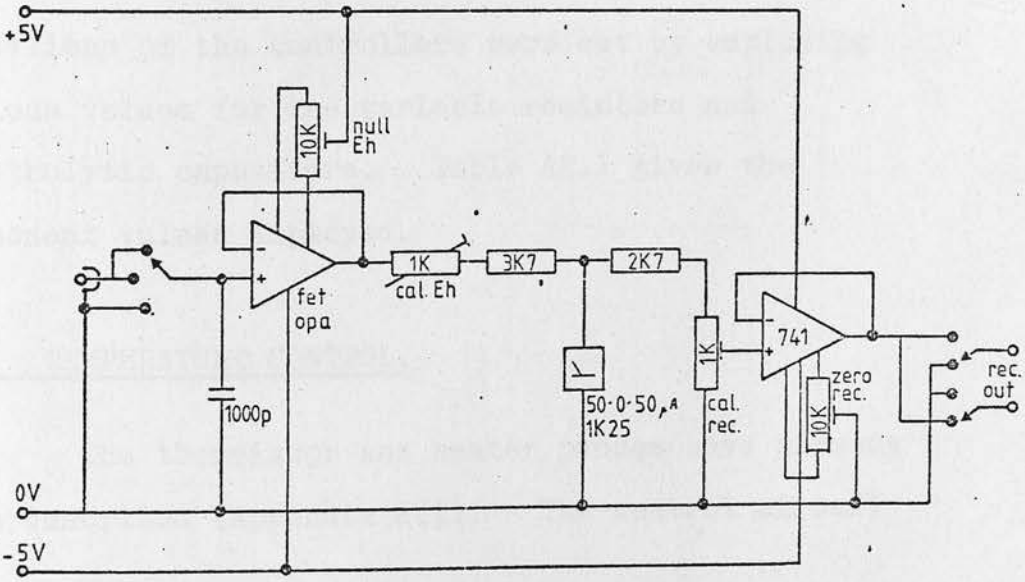


Fig. A.2.12 Eh meter circuit.

screened case behind the meter panel.

The meter circuits employed FET input integrated circuit operational amplifiers (Figures A2.11 and A2.12), that for the Eh meter being arranged as a voltage follower with unity gain, whilst in the case of the pH meter gain was set at approximately 2. The pH meter provided a signal for the control unit mounted in the rack above the pH/Eh meter panel.

Both circuits incorporated voltage-follower amplifiers and voltage dividing resistors to drive the appropriate channels of the chart recorder (Appendix 2.L). The instruments were calibrated using a reference mV source.

2.H PUMP CONTROLS.

The pump control circuit is described in a reprint included in the insert pocket.

The time constants for the "add" and "hold" conditions of the controllers were set by employing various values for the variable resistors and electrolytic capacitors. Table A2.1 gives the component values employed.

2.I TEMPERATURE CONTROL.

The thermistor and heater probes have already been described (Appendix 2.A). The control circuit

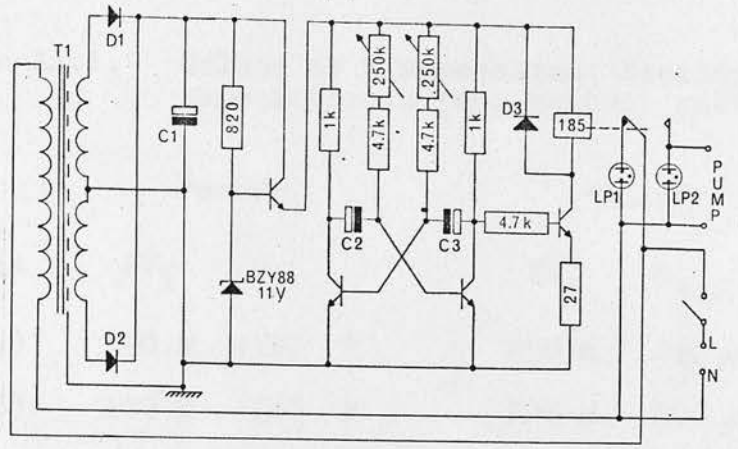


Fig. A.2.13 Pump control unit circuit (see text)

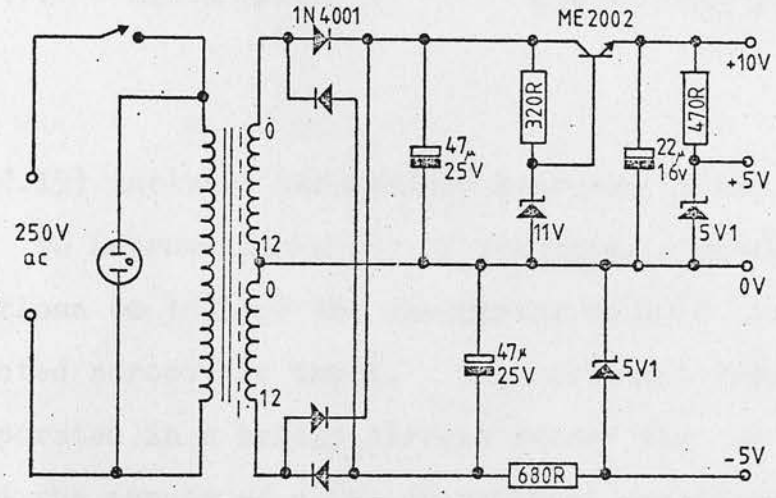


Fig. A.2.14 Power supply for pH and temperature control.

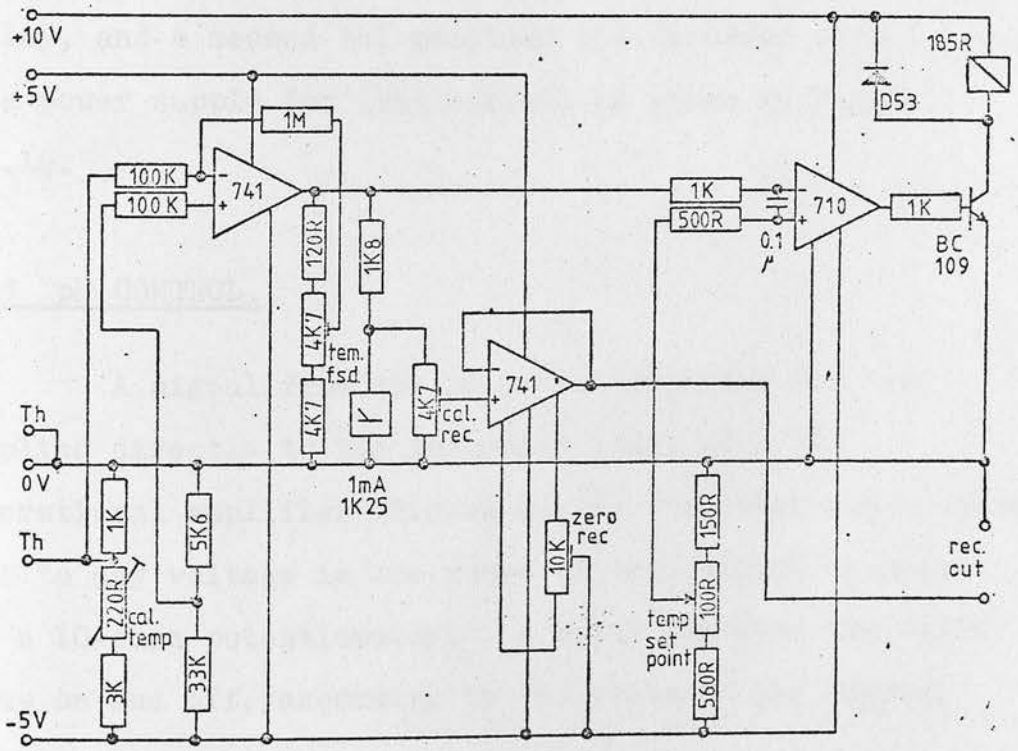


Fig. A.2.15 Temperature control circuit.

Table A2.1. Values of time-constant resistors and capacitors in pump-control units.

	"add"		"hold"	
control unit	RV_1	C_2	RV_2	C_3
addition (4)	100 k	μ100 F	250 k	200 μF
feed (5)	100 k	μ100 F	250 k	200 μF
dialysis (6)	100 k	μ100 F	250 k	100 μF
antifoam (7)	250 k	μ100 F	250 k	100 μF

(Figure A2.15) included temperature measurement and display. To improve linearity of response, a resistor of value close to that of the thermistor at 40°C (ie 1 k) was connected across the input. The resultant reistance was incorporated in a bridge circuit across the -5 V supply and the inputs of a 741 operational amplifier. A 710 comparitor provided temperature control via a relay, and a second 741 provided the recorder output. The power supply for this circuit is shown in Figure A2.14.

2.J pH CONTROL.

A signal from the pH meter (Appendix 2.G) was applied directly to the inverting input of a 710 operational amplifier (Figure A2.16) the other input being set to any voltage in the range of this signal by means of a 10-turn potentiometer. A relay switched the buffer pump on and off, according to the state of the inputs,

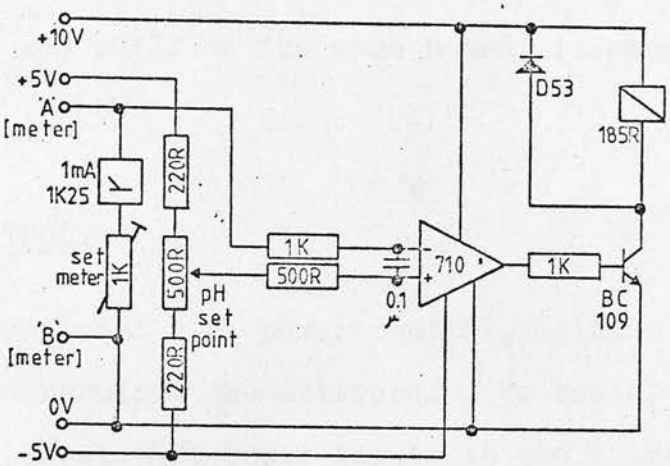


Fig. A.2.16 pH control circuit.

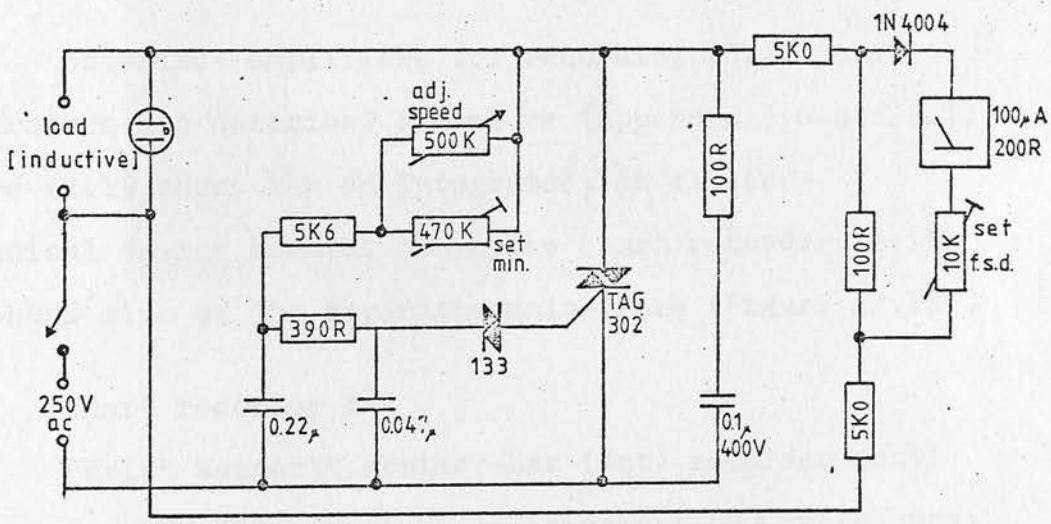


Fig. A.2.17 Stirr control circuit.

via a BC 109 transistor. The power supply for this circuit, which was built on the same board, is shown in Figure A2.14.

2.K STIR CONTROL.

A conventional a.c. power control, using a triac, was employed to regulate the stirrers. To assist the application of consistant power inputs to the stirrers, the outputs from the control circuits were voltage divided, rectified and applied to the panel meters (Figure A2.17).

2.L CHART RECORDER AND INTERFACES.

Interface amplifiers for recording pH, Eh and temperature are described elsewhere (Appendix 2.G and 2.I). Figure A2.19 shows the pH integrater, an electro-mechanical device mounted above the chart recorder on the left-hand side of the apparatus main-frame (Figure A2.18).

Chart recorder :

"Erich Weinert" striker-bar (dot) recorder model no. FPM 120. Moving coil display; 40Ω resistance; 8 mV F.S.D.; 1.5% accuracy. Six channels, cycling rate 20 seconds; chart speed 20 mm/h; chart width 120 mm.

2.M SLURRY PREPARATION.

1. Initial slurry prepared in "Alexander" homogeniser

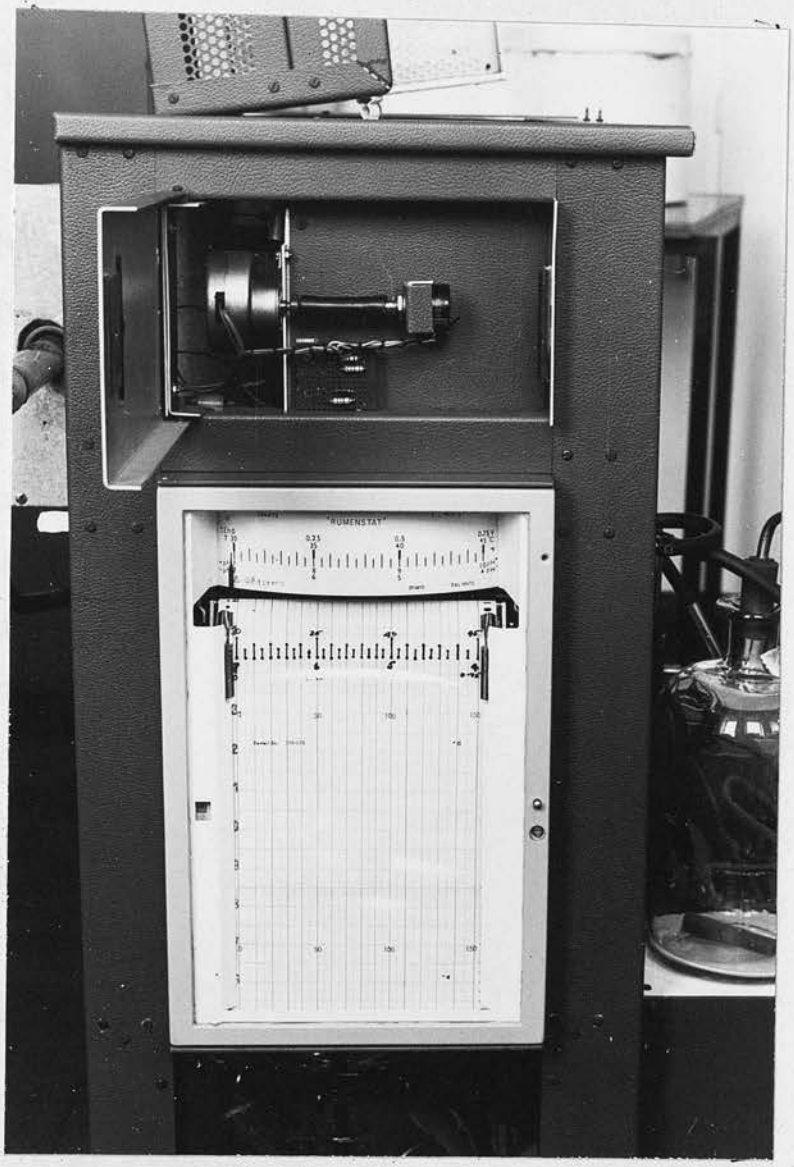


Fig. A.2.18 Recorder and pH integrater.

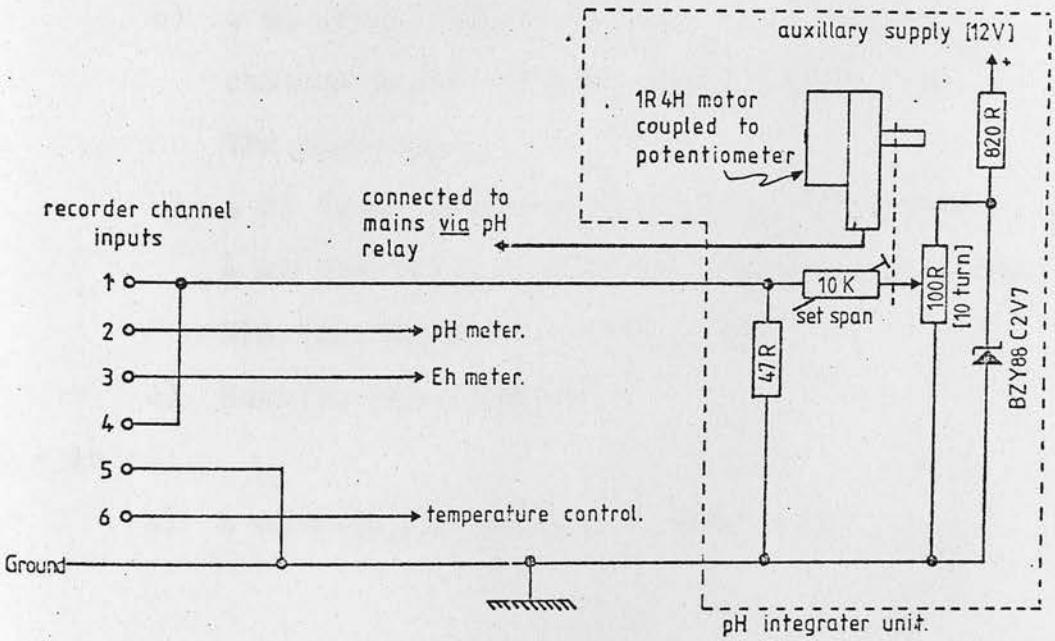


Fig. A.2.19 Chart recorder channel connections and pH integrater unit.

to yield a DM $\geqslant$4% (Quantities calculated from silage DM).

2. Exact DM of initial slurry determined.
3. pH adjusted to 3.5 with HCl.
4. DM adjusted to 3.97 - 3.98 by addition of water.
5. 0.025% high-substitution methyl cellulose added whilst stirring.
6. Stirring continued and repeated using "split disc" impellor ("Gallenkamp" part no. SS 552) or laboratory stirrer ("Griffin" part no. S 37-954).
7. Large residual fibre particles removed from slots of stirring disc until no more appear.
8. Entire slurry checked for homogenicity by passing through 3.2 mm ($\frac{1}{8}$") orifice.

2.N SAMPLING SCHEDULE.

I (first sample)

a) 5 ml from culture for NH_3-N determination (see Appendix 1.E)

b) 4 ml from culture vessel, added to 4 ml chilled metaphosphoric acid reagent, for VFA analysis

c) 4 ml from dialysate discharge line, added to 4 ml chilled metaphosphoric acid reagent for VFA (see Appendix 1.F)

d) Machine data recorded

I + 1h

a) 4 ml from culture vessel plus 4 ml

formalised 30% glycerol (stored at -20°C for total counts)

b) 4 ml from culture stored at -20°C

c) Machine data recorded

d) Media and dilutions prepared for cultural counts

I + 2h

a) 1 ml from culture vessel; initial dilution for cultural counts

b) Machine data recorded

c) Cultural count dilutions and inoculations carried out

I + 6h

a) NH_3-N determinations titrated

b) Machine inspected and reservoirs replenished as necessary. Recorder chart checked. Pump element tubes, furnace combustion tube and gas cylinders checked and replaced as necessary

c) Nominal pump-delivery rates in preceeding 24 h calculated and control adjustment made if necessary.

2.0 GENERAL DIMENSIONS AND OTHER INFORMATION.

Figure A2.20 shows the overall dimensions of the Rumenstat. Reference should also be made to Figure 16 (page 117).

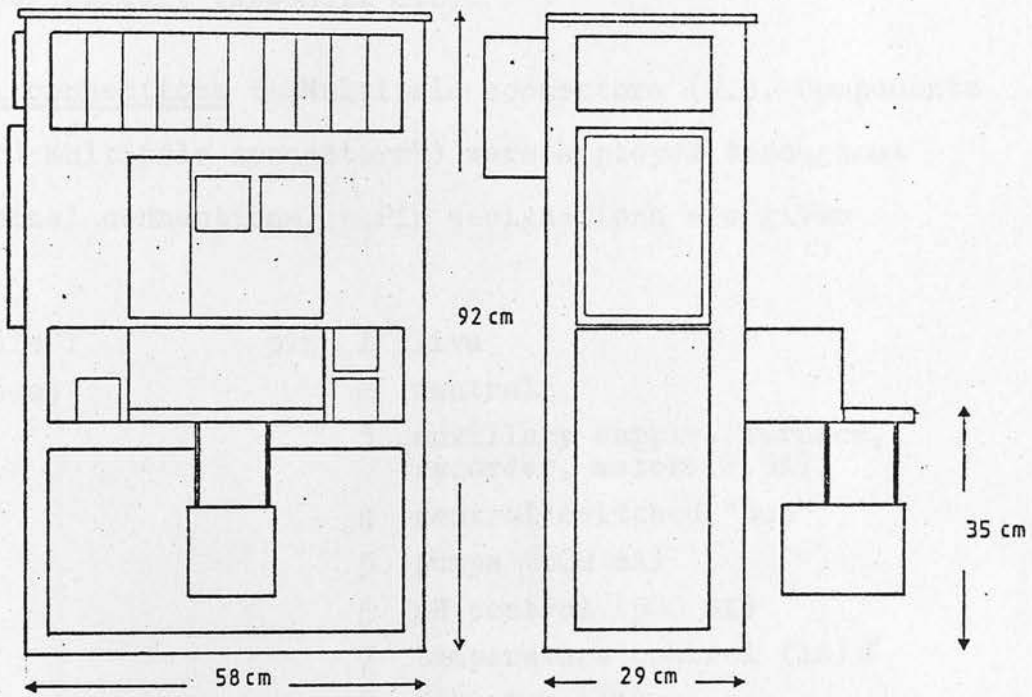


Fig. A.2.20 General dimensions and layout of Rumenstat.

Auxillary supply : An unregulated 12V DC (nominal) power supply unit was incorporated within the main-frame, behind the furnace control panel. This supply was switched on with the main power switch and provided for illumination of the gas flow gauges and the culture vessel inspection lamp. This supply also powered the pH integrator circuit (Appendix 2.L).

Internal connections : Multipole connectors (R.S. Components "standard multipole connectors") were employed throughout for internal connections. Pin designations are given below :

Module 1 (fuses)	pin	1	live
		2	neutral
		3	auxillary supply, furnace, recorder, meters (2.5A)
		4	neutral switched "out"
		5	pumps (500 mA)
		6	pH control (500 mA)
		7	temperature control (1A)
		8	stirring (1A)
Module 2 (pH control)	pin	1	integrator (relay '2' N.O.)
		2	integrator (relay '2' COMMON)
		3	meter 0V
		4	meter signal
		5	live } 240V a.c.
		6	neutral }
		7	pump (control signal)
		8	NC
Module 3 (temperature)	pin	1	thermistor }
		2	thermistor }
		3	recorder }
		4	recorder }

	pin 5	live	240V a.c.
	6	neutral	
	7	heater (control signal)	
	8	NC	
Modules 4,5,6,7 (pumps)	pin 1	live	240V a.c.
	2	neutral	
	3	pump (control signal)	
	4	NC	
Modules 8 & 9 (stir control)	pin 1	live	240V a.c.
	2	neutral	
	3	live	control signal
	4	neutral	
Module 10	-		
Module 11 (pH & Eh meters)	pin 1	live	240V a.c.
	2	neutral	
	3	V - (5V)	
	4	pH meter signal to control	
	5	V + (5V)	
	6	NC	
	7	OV (ground)	
	8	NC	
	9	recorder Eh	
	10	recorder Eh	
	11	recorder pH	
	12	recorder pH	

Storage reservoirs : "Quickfit" F.V. series flasks, with single-port covers, of 5 l capacity (effluent and dialysis solution) and 2 l capacity (buffer solution).

Culture vessel : 600 ml "Pyrex" squat-form beaker enclosed in a PVC jacket 11 cm diameter and 12 cm long

with a 90 x 35 mm inspection window.

Pumps : "Peripump" minature peristaltic pumps with output speeds and element tubes as detailed below :

	element tube	speed
dialysis pump	2.5 mm Si rubber	10 rpm
buffer pump	2.5 mm Si rubber	10 rpm
antifoam/addition pump	1.5 mm butyl rubber	6 rpm

Pump element tubes were protected from pump rotor abrasion by a strip of PTFE impregnated glass fibre cloth 12 mm wide. The strip was attached to the input nipple by a plastic rachet tie securing the tube.

2.P SUPPLIERS.

Activion Glass Ltd.,
Mitchell Hall,
Kinglassie,
Fife.
— pH electrodes, Eh electrodes, reference electrodes

A. Gallenkamp & Co. Ltd.,
P.O. Box 290,
Technico House,
Christophen Street,
London, EC2P 2ER.
— Laboratory stirrers, Glassware etc.

Combined Electronic Services Ltd.,
604 Purley Way,
Waddon,
Croydon, CR9 4DR.
— Drive belts

Crouzet (U.K.) Ltd., Brentford, Middlesex.	Synchronous motors Induction motors
Esco (Rubber) ltd., 14-16 Great Portland Street, London, W1N 5AB.	Silicone rubber tubes Butyl rubber tubes Stoppers
G.A. Playton, (Flowbits) Basingstoke, RG21 2XD.	Gas lfow gauges Valves
Glen Creston, 37 The Broadway, Stamore, Middlesex, HA7 4DL.	W.A.B. peristaltic pump (media dispenser)
I.T.T. Electronic Services, Edinburgh Way, Harlow, Essex.	Switches, knobs, relays, Semiconductors
National Research and Development Corporation Ltd., P.O. Box 236, Kingsgate House, 66/74 Victoria Street, London, SW1E 6SC.	Patentees of "Alexander" homogeniser
R.S. Components Ltd., P.O. Box 427, 13-17 Epworth Street, London, EC2P sHA.	Wire, cables, transformers, passive components,sundray hardware etc.
Schuco Scientific Ltd., Halliwich Court Place, Woodhouse Road, London, N.12	Peripumps, plastic rachet ties, glass fibre tape

Sparmac Ltd., 77 Dundonald Road, Troon, Ayrshire.	Makers of "Alexander" homogeniser
T.E.M. Sales Ltd., Gatwick Road, Cranley, Sussex, RH10 2RG.	Chart recorder and accessories

APPENDIX 3.

(Detailed Results)

3.A In Vivo Experiments.

3.A.1 Group 1 silages.

silage	sheep	DMI[1] (g)	Counts ($X10^5$), means of 3 tubes. starch	gelatin	cellulose	NH_3-N (mg/l)
control	437	920	22.0	35.0	1.44	277
	447	895	13.0	37.5	1.21	353
	414	956	15.1	45.4	1.47	438
formic acid/ acetic acid	449	992	4.55	42.0	3.55	277
	434	983	5.90	39.0	2.40	406
	434[2]	992	7.60	23.0	5.13	456
f/dehyde/ acetic acid	448	1036	42.0	13.5	22.9	293
	88	949	20.6	15.2	18.3	256
	409	1040	16.9	21.0	29,5	255

1. Measured as oven DM and adjusted for toluene DM.

2. Sheep 434 sampled twice on different days.

/ f/dehyde = formaldehyde /

3.A.2 Group 2 silages.

Code: A = control
B = wilted
C = fresh/formic acid
D = wilted/formic acid

	sheep:	414	434	409	447	437	448	88	449
i	DMI (g/d)								
	A	620	981	554	439	659	917	625	690
	B	697	1054	783	540	946	1154	809	931
	C	659	617	855	438	807	943	616	757
	D	782	872	922	726	922	1090	804	943
ii	counts on starch ($X10^5$), means of 3 tubes.								
	A	1.56	1.54	1.55	1.59	1.64	1.62	1.54	1.52
	B	1.92	2.48	1.04	2.24	1.50	2.42	1.12	1.18
	C	2.04	1.90	1.90	2.14	2.58	1.28	1.94	1.58
	D	2.68	4.38	3.32	3.74	3.84	3.19	3.05	1.78
iii	counts on gelatin ($X10^5$), means of 3 tubes.								
	A	1.94	2.74	3.54	1.67	2.75	2.90	2.15	3.60
	B	1.60	2.64	3.00	1.13	1.84	2.24	1.90	1.94
	C	3.80	4.10	4.70	3.10	3.60	3.40	4.0	5.42
	D	8.70	15.10	6.12	8.40	11.67	12.70	12.90	6.47
iv	counts on cellulose ($X10^5$), means of 3 tubes.								
	A	1.40	2.04	2.29	2.54	2.05	4.45	1.12	2.90
	B	1.35	1.35	1.41	1.36	1.36	2.03	1.18	1.45
	C	1.68	2.04	2.06	1.28	3.19	2.00	1.90	2.24
	D	3.39	3.14	3.13	3.65	4.0	3.70	3.48	3.34

3.A.3 Group 3 silages.

Code: A = control
B = formaldehyde/formic acid
C = formaldehyde/H_2SO_4
D = formaldehyde

sheep:	88	409	414	434	437	447	449	680
i DMI (g/d)								
A	857	843	973	1059	1069	996	967	470
B	1032	1047	1054	1038	1056	1038	1058	687
C	888	1037	1018	1063	1081	1062	1086	648
D	1046	1058	982	1015	1061	1062	1031	805
ii counts on starch ($X10^5$), means of 3 tubes.								
A	1.24	2.62	6.6	3.84	2.08	2.82	8.8	1.20
B	9.2	19.8	11.0	9.2	13.2	10.8	7.2	9.6
C	23.8	30.0	9.2	8.4	8.6	13.4	17.0	19.4
D	2.96	2.88	3.70	3.84	1.60	2.38	4.32	3.96
iii counts on gelatin ($X10^5$), means of 3 tubes.								
A	18.0	21.4	2.28	6.36	2.60	2.92	17.2	7.2
B	1.62	3.40	3.5	0.98	1.88	2.32	2.6	1.90
C	12.0	5.6	4.88	10.8	5.0	6.80	10.0	9.00
D	1.18	2.72	3.20	1.88	1.88	2.38	4.94	4.58
iv counts on cellulose ($X10^5$), means of 3 tubes.								
A	1.10	1.42	2.14	1.74	6.00	1.08	1.28	1.04
B	17.2	6.80	5.60	4.2	11.4	18.2	6.8	24.4
C	20.2	28.2	26.4	6.8	5.4	14.2	13.2	16.2
D	10.8	16.4	12.9	10.6	10.6	12.0	11.9	12.3

3.B In vitro Experiments.

3.B.1 Group 1 silages.

i Control silage (Run 14)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	2	6	11	16	21	26
ET	20	44	68	92	117	130
mg/l	237	167	180	181	206	218
S.D.	6.18	16.9	10.7	3.78	2.90	7.57

b) VFA (mM/l)

sample 4, ET 26

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.29	2.87	0.16	2.48	0.54	0.32	0.17
$C_d \times F_d$	33.7	10.5	0.59	8.99	1.97	1.15	0.63
C_p	26.8	8.91	1.07	7.52	1.55	0.34	0.44
C_t	60.5	19.4	1.66	16.5	3.52	1.49	1.07
$M\%C_t$	58.1	18.6	1.60	15.8	3.38	1.44	1.03
$C_t \times F_f$	89.5	28.6	2.46	24.4	5.20	2.21	1.59

sample 8, ET 47

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	7.22	3.38	0.14	2.52	0.36	0.26	0.23
$C_d \times F_d$	26.2	12.3	0.50	9.16	1.31	0.96	0.84
C_p	34.0	9.68	1.14	8.67	1.38	0.68	0.89
C_t	60.3	21.9	1.64	17.8	2.69	1.65	1.78
$M\%C_t$	55.9	20.4	1.52	16.5	2.50	1.53	1.60
$C_t \times F_f$	89.1	32.4	2.42	26.3	3.98	2.43	2.55

Sample 13, ET 95

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	18.7	4.84	0.16	3.17	0.45	0.32	0.23
$C_d \times F_d$	68.0	17.5	0.59	11.5	1.64	1.15	0.84
C_p	48.2	16.1	1.07	10.6	1.30	0.77	0.88
C_t	116	33.7	1.66	22.1	2.94	1.92	1.73
$M\%C_t$	64.5	18.7	0.92	12.3	1.63	1.07	0.96
$C_t \times F_f$	171	49.8	2.46	32.7	4.35	2.84	2.55

sample 18, ET 118

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	18.0	7.36	0.27	6.26	0.59	0.64	0.35
$C_d \times F_d$	65.5	26.7	0.99	22.7	2.13	2.31	1.26
C_p	47.5	17.5	1.41	14.2	1.22	1.11	0.74
C_t	113	44.2	2.40	36.9	3.36	3.42	2.00
$M\%C_t$	55.0	21.5	1.17	18.0	1.64	1.66	0.97
$C_t \times F_f$	167	65.2	3.54	54.5	4.96	5.06	2.96

sample 24, ET 141

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	18.1	7.08	0.14	5.91	0.56	0.67	0.33
$C_d \times F_d$	65.7	25.7	0.49	21.5	2.04	2.46	1.20
C_p	45.8	18.6	1.30	13.7	1.29	1.00	0.69
C_t	111	44.3	1.79	35.2	3.33	3.45	1.90
$M\%C_t$	55.3	21.9	0.89	17.5	1.65	1.72	0.94
$C_t \times F_f$	164	65.5	2.65	52.0	4.92	5.11	2.81

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	10	15	20	23	30
ET	49	73	97	118	132
starch	0.89	3.03	10.4	27.4	28.6
S.D.	0.04	0.22	0.60	4.29	1.74
gelatin	0.59	0.59	3.31	26.2	25.6
S.D.	0.17	0.16	0.31	5.38	0.58
cellulose	0.73	0.82	22.2	41.0	40.0
S.D.	0.09	0.11	2.80	2.16	2.00

d) Total counts (bacteria X 10^8, protozoa X 10^4, means of 5 counts, not adjusted for F_f)

sample	3	7	12	17	22	27
ET	21	45	69	93	117	136
bacteria	1.58	0.72	2.2	2.16	2.12	1.94
S.D.	0.55	0.31	0.69	0.67	0.90	0.55
protozoa	5.25	2.69	2.49	1.38	2.12	3.17
S.D.	1.45	0.48	0.44	0.56	0.56	0.69

ii Formic acid/acetic acid treated silage (Run 12)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	2	7	11	16	21
ET	20	44	69	94	119
mg/l	125	138	156	192	191
S.D.	1.60	0.92	3.36	2.06	2.31

b) VFA (mM/l)

sample 4, ET 22

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	18.4	4.32	0.19	2.89	0.59	0.37	0.17
$C_d \times F_d$	57.3	13.5	0.59	9.01	1.84	1.15	0.54
C_p	47.0	11.8	0.89	8.22	1.66	0.72	0.44
C_t	104	24.3	1.49	17.2	3.49	1.87	0.98
$M\%C_t$	67.9	15.8	0.97	11.2	2.27	1.22	0.64
$C_t \times F_f$	153	35.8	2.19	25.4	5.15	2.76	1.45

sample 8, ET 50

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.3	2.78	0.08	2.45	0.45	0.21	0.41
$C_d \times F_d$	32.1	8.67	0.25	7.66	1.41	0.66	1.26
C_p	42.0	9.65	1.08	6.92	1.74	0.72	0.59
C_t	74.1	18.3	1.34	14.6	3.15	1.38	1.86
$M\%C_t$	64.6	15.9	1.16	12.7	2.75	1.20	1.62
$C_t \times F_f$	109	26.9	1.97	21.5	4.67	1.77	2.73

sample 13, ET 70

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.8	3.24	0.11	2.94	0.46	0.21	0.17
$C_d \times F_d$	36.8	10.1	0.34	9.16	1.27	0.66	0.54
C_p	36.8	5.59	1.08	4.41	1.24	0.32	0.44
C_t	73.7	15.7	1.42	13.6	2.52	0.98	0.98
$M\%C_t$	67.7	14.4	1.31	14.5	2.31	0.90	0.90
$C_t \times F_f$	108	23.1	2.09	19.9	3.71	1.44	1.45

sample 18, ET 96

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.3	2.78	0.08	2.45	0.45	0.21	0.41
$C_d \times F_d$	32.1	8.67	0.25	7.66	1.41	0.66	1.26
C_p	31.9	5.01	0.95	3.98	1.24	0.40	0.48
C_t	64.0	13.7	1.21	11.6	2.66	1.06	1.75
$M\%C_t$	61.5	13.1	1.16	11.2	2.55	1.02	1.67
$C_t \times F_f$	94.3	20.1	1.78	17.1	3.91	1.56	2.57

sample 23, ET 110

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	13.1	2.73	0.11	2.28	0.36	0.21	0.35
$C_d \times F_d$	40.8	8.53	0.34	7.12	1.13	0.66	1.08
C_p	31.0	5.05	0.83	4.20	1.16	0.40	0.43
C_t	71.8	13.7	1.16	11.3	2.29	1.06	1.51
$M\%C_t$	69.8	13.3	1.13	11.0	2.22	1.03	1.47
$C_t \times F_f$	105	20.1	1.72	16.7	3.37	1.56	2.23

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	9	14	20	24
ET	50	71	99	120
starch	15.94	12.46	5.06	14.57
S.D.	0.30	2.80	1.10	0.42
gelatin	7.86	10.66	5.40	13.26
S.D.	1.30	1.60	-	3.32
cellulose	15.20	9.00	6.12	17.14
S.D.	2.8	2.00	2.30	0.62

d) Total counts (bacteria X 10^8, protozoa X 10^4, means of 5 counts, not adjusted for F_f)

sample	5	10	15	19	25
ET	23	51	70	97	121
bacteria	2.86	2.08	1.72	1.64	1.50
S.D.	0.69	0.30	0.19	0.44	0.30
protozoa	7.10	5.31	3.84	3.52	3.46
S.D.	1.00	0.90	0.84	0.74	0.70

iii Formaldehyde/acetic acid treated silage (Run 10)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	1	5	10	15	20	25
ET	22	44	92	118	141	150
mg/l	135	95.5	NR	79.5	81.6	82
S.D.	0.30	1.81	-	1.30	0.38	0.97

b) VFA (mM/l)

sample 3, ET 26

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	13.4	1.70	0.08	1.61	0.22	0.21	0.12
$C_d \times F_d$	41.1	5.21	0.25	4.92	0.69	0.65	0.35
C_p	30.2	5.09	0.58	4.93	0.86	0.56	0.27
C_t	71.3	10.3	0.83	9.85	1.56	1.21	0.62
$M\%C_t$	74.5	10.7	0.34	10.3	1.62	1.27	0.65
$C_t \times F_f$	105	15.2	1.23	14.6	2.30	1.79	0.92

sample 8, ET 47

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	12.5	1.15	0.05	1.12	0.09	0.16	0.12
$C_d \times F_d$	38.4	3.52	0.17	3.44	0.28	0.49	0.35
C_p	22.7	3.66	0.23	3.56	0.31	0.28	0.27
C_t	61.0	7.28	0.40	7.00	0.59	0.77	0.62
$M\%C_t$	78.5	9.37	0.52	9.0	0.76	0.99	0.80
$C_t \times F_f$	90.3	10.7	0.59	10.3	0.87	1.14	0.92

sample 13, ET 95

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.3	1.19	0.05	1.13	0.14	0.21	0.35
$C_d \times F_d$	31.4	3.66	0.16	3.46	0.42	0.65	1.06
C_p	24.6	4.76	0.46	3.64	0.47	0.49	1.46
C_t	56.1	8.42	0.63	7.1	0.89	1.14	2.53
$M\%C_t$	73.0	10.9	0.82	9.24	1.12	1.49	3.29
$C_t \times F_f$	82.9	12.4	0.94	10.5	1.31	1.69	3.74

sample 17, ET 118

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.8	1.43	0.55	0.96	0.14	0.16	0.40
$C_d \times F_d$	33.1	4.36	0.17	2.93	0.42	0.49	1.24
C_p	27.0	5.09	0.76	4.39	0.63	0.56	1.73
C_t	60.1	9.46	0.92	7.33	1.04	1.05	2.97
$M\%C_t$	72.5	11.4	1.11	8.83	1.26	1.27	3.58
$C_t \times F_f$	88.9	13.9	1.37	10.8	1.54	1.55	4.39

sample 21, ET 141

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	12.6	1.52	0.55	1.09	0.14	0.20	0.41
$C_d \times F_d$	38.5	4.65	0.17	3.33	0.42	0.62	1.24
C_p	26.6	4.43	0.71	3.87	0.58	0.41	1.73
C_t	65.1	8.07	0.87	7.19	0.99	1.02	2.97
$M\%C_t$	75.5	9.36	1.01	8.34	1.15	1.18	3.44
$C_t \times F_f$	96.3	11.9	1.29	10.6	1.47	1.51	4.39

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	7	12	19	22
ET	46	94	122	143
starch	11.4	22.6	22.86	19.34
S.D.	1.64	-	4.57	1.92
gelatin	10.0	2.0	3.08	1.62
S.D.	0.52	0.14	0.27	0.07
cellulose	2.20	2.97	3.11	2.17
S.D.	0.16	0.09	0.08	0.07

d) Total counts (bacteria X 10^8, protozoa X 10^4, means of 5 counts, not adjusted for F_f)

sample	2	6	11	16	19	22
ET	23	46	93	118	122	143
bacteria	1.04	1.44	1.66	1.78	2.52	3.24
S.D.	0.45	0.39	0.40	0.31	0.63	0.84
protozoa	2.50	1.18	2.24	3.55	3.46	4.77
S.D.	0.92	0.63	0.72	0.31	0.24	0.59

3.B.2 Group 2 silages.

i Control (Run 15)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	2	6	11	16	21
ET	9	38	62	86	110
mg/l	246	244	216	220	214
S.D.	1.09	3.87	1.01	2.56	0.55

b) VFA (mM/l)

sample 4, ET 16

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	13.7	5.23	0.29	2.96	0.51	0.43	0.14
$C_d \times F_d$	61.5	23.5	13.3	13.3	2.27	1.95	0.63
C_p	32.2	12.6	0.76	11.9	1.49	0.86	0.41
C_t	93.7	36.1	14.1	25.2	3.76	2.81	1.04
$M\%C_t$	53.0	20.4	7.97	14.2	2.13	1.59	0.58
$C_t \times F_f$	130	50.1	19.6	35.0	5.22	3.98	1.43

sample 8, ET 40

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.27	3.18	0.15	1.44	2.53	0.22	0.09
$C_d \times F_d$	41.6	1.43	0.66	6.46	1.14	0.97	0.42
C_p	26.5	9.19	0.52	7.89	1.02	0.50	0.30
C_t	68.1	10.6	1.19	14.3	2.16	1.47	0.73
$M\%C_t$	69.0	10.7	1.20	14.5	2.18	1.49	0.74
$C_t \times F_f$	94.7	14.7	1.65	19.9	2.99	2.04	1.01

sample 13, ET 64

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.96	3.23	0.11	1.30	0.25	0.22	0.94
$C_d \times F_d$	44.7	14.5	0.49	5.84	1.14	0.97	0.42
C_p	18.0	7.91	0.41	5.98	0.78	0.42	0.30
C_t	62.8	22.4	0.91	11.8	1.92	1.40	0.73
$M\%C_t$	61.6	21.8	0.89	11.6	1.88	1.37	0.71
$C_t \times F_f$	87.2	31.1	1.26	16.4	2.67	1.95	1.01

sample 18, ET 88

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.13	3.04	0.11	0.93	0.21	0.16	0.09
$C_d \times F_d$	41.0	13.6	0.50	4.18	0.95	0.73	0.42
C_p	25.4	7.69	0.52	6.93	0.78	0.43	0.30
C_t	66.5	21.3	1.03	11.1	1.73	1.16	0.72
$M\%C_t$	64.1	20.6	0.99	10.7	1.67	1.12	0.70
$C_t \times F_f$	92.4	29.6	1.43	15.4	2.41	1.61	1.01

sample 23, ET 112

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	13.3	3.69	0.14	1.67	0.25	0.27	0.09
$C_d \times F_d$	59.9	16.6	0.66	7.49	1.14	1.22	0.42
C_p	25.1	8.25	0.52	7.17	0.63	0.50	0.30
C_t	85.1	24.8	1.19	14.6	1.76	1.72	0.73
$M\%C_t$	65.5	19.1	0.91	11.3	1.36	1.33	0.56
$C_t \times F_f$	118	34.5	1.65	20.4	2.45	2.39	1.01

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	9	14	20	24
ET	41	64	89	112
starch	8.4	11.2	10.1	11.9
S.D.	0.8	1.4	0.64	1.59
gelatin	0.52	0.47	0.51	0.51
S.D.	0.14	0.10	0.08	0.04
cellulose	11.6	16.2	15.8	15.3
S.D.	0.20	1.84	0.22	0.76

d) Total counts (bacteria X 10^8, protozoa X 10^4, means of 5 counts, not adjusted for F_f)

sample	3	7	12	17	22
ET	15	39	63	87	111
bacteria	1.8	1.28	1.26	1.54	1.64
S.D.	0.47	0.50	0.40	0.51	0.36
protozoa	2.94	2.85	2.30	2.11	2.80
S.D.	0.94	0.48	0.52	0.55	0.43

ii Wilted (Run 13)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	2	6	11	16	21
ET	18	43	67	91	116
mg/l	229	194	265	254	259
S.D.	3.41	8.83	5.89	5.89	4.20

b) VFA (mM/l)

sample 4, ET 20

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	16.0	4.23	0.22	3.22	0.42	0.49	0.09
$C_d \times F_d$	69.7	18.5	0.97	13.9	1.83	2.12	0.41
C_p	31.3	9.50	0.67	7.59	1.37	1.12	0.45
C_t	101	28.0	1.63	21.6	3.21	3.25	0.86
$M\%C_t$	62.9	17.5	1.02	13.4	1.99	2.03	0.38
$C_t \times F_f$	135	37.5	2.19	28.8	4.28	4.34	1.15

sample 8, ET 45

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.3	1.32	0.07	1.24	0.29	0.22	0.14
$C_d \times F_d$	36.1	5.77	0.32	5.39	1.28	0.94	0.63
C_p	28.5	5.14	0.44	5.22	1.51	0.78	0.79
C_t	64.5	10.9	0.77	10.6	2.78	1.72	1.43
$M\%C_t$	69.6	11.7	0.83	11.4	3.00	1.86	1.54
$C_t \times F_f$	86.2	14.6	1.03	14.2	3.72	2.30	1.90

sample 13, ET 69

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.55	1.28	0.11	1.33	0.25	0.22	0.18
$C_d \times F_d$	41.5	5.56	0.48	5.79	0.66	0.94	0.81
C_p	31.8	8.88	0.37	5.22	1.32	0.78	0.91
C_t	73.3	14.4	0.85	11.0	1.98	1.72	1.72
$M\%C_t$	69.8	13.8	0.82	10.5	1.88	1.64	1.63
$C_t \times F_f$	97.9	19.3	1.14	14.7	2.65	2.30	2.29

sample 18, ET 93

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	17.4	4.22	0.15	1.98	0.29	0.33	0.18
$C_d \times F_d$	75.8	18.4	0.64	8.59	1.28	1.42	0.81
C_p	38.9	10.8	0.37	5.76	1.03	0.95	0.79
C_t	114	29.2	1.01	14.4	2.32	2.37	1.60
$M\%C_t$	69.2	17.6	0.61	8.67	1.39	1.43	0.97
$C_t \times F_f$	153	38.9	1.35	19.2	3.09	3.17	2.14

sample 23, ET 117

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	15.2	2.42	0.15	1.93	0.34	0.38	0.18
$C_d \times F_d$	66.2	10.5	0.64	8.39	1.47	1.65	0.81
C_p	33.2	9.90	0.52	5.12	1.13	0.87	0.56
C_t	99.4	20.4	1.16	13.5	2.59	2.52	1.38
$M\%C_t$	70.5	14.5	0.83	9.59	1.84	1.78	0.97
$C_t \times F_f$	132	27.3	1.55	18.1	3.47	3.37	1.84

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	9	14	19	24
ET	46	69	94	118
starch	0.69	0.63	0.98	1.34
S.D.	0.17	0.21	0.16	0.10
gelatin	0.51	0.67	0.63	0.63
S.D.0.08	0.05	0.08	0.12	
cellulose	0.53	0.37	1.40	1.36
S.D.	0.13	0.10	0.08	0.14

d) Total counts (bacteria X 10^8, protozoa X 10^4; means of 5 counts, not adjusted for F_f)

sample	3	7	12	17	22
ET	19	44	68	92	117
bacteria	1.5	1.14	1.32	1.74	1.10
S.D.	0.35	0.24	0.34	0.51	0.38
protozoa	3.81	3.14	0.93	0.74	0.48
S.D.	1.02	0.42	0.13	0.33	0.16

iii Fresh/formic (Run 17)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	2	6	11	16	22
ET	20	44	68	92	117
mg/l	335	288	267	260	256
S.D.	8.41	6.35	3.49	3.87	2.69

b) VFA (mM/l)

sample 4, ET 22

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.07	2.37	0.22	1.93	0.84	0.27	0.14
$C_d \times F_d$	41.0	10.7	1.01	7.85	3.82	1.24	0.63
C_p	29.7	9.19	0.87	4.21	1.53	0.71	0.41
C_t	70.8	19.0	1.88	12.9	5.35	1.95	1.04
$M\%C_t$	62.1	17.5	1.65	11.4	4.70	1.71	0.91
$C_t \times F_f$	95.8	25.9	2.55	17.5	7.24	2.64	1.41

sample 9, ET 46

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.09	1.54	0.22	1.16	1.05	0.22	0.33
$C_d \times F_d$	36.7	6.98	1.01	5.23	4.78	0.98	1.45
C_p	21.3	4.58	0.37	3.99	1.33	0.61	0.47
C_t	58.0	11.6	1.38	9.22	6.11	1.59	1.92
$M\%C_t$	64.6	15.6	1.87	12.5	8.26	2.15	2.68
$C_t \times F_f$	78.5	15.6	1.86	12.4	8.26	2.15	2.68

sample 14, ET 70

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.91	1.78	0.19	1.66	0.84	0.22	0.33
$C_d \times F_d$	40.4	8.06	0.84	7.50	3.82	0.98	1.48
C_p	19.4	4.58	0.97	3.06	1.29	0.61	0.36
C_t	59.8	12.6	1.81	10.5	5.11	1.59	1.84
$M\%C_t$	64.1	13.5	1.94	11.3	5.48	1.70	1.97
$C_t \times F_f$	80.9	16.6	2.45	14.3	6.92	2.15	2.49

sample 19, ET 94

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	6.25	1.45	0.19	1.02	0.46	0.22	0.23
$C_d \times F_d$	29.8	6.29	0.81	4.68	2.02	0.94	1.02
C_p	16.6	3.74	0.67	2.45	1.49	0.36	0.71
C_t	46.4	10.0	1.48	7.13	3.51	1.30	1.73
$M\%C_t$	64.8	14.0	2.06	9.96	4.89	1.82	2.50
$C_t \times F_f$	62.79	13.6	2.00	9.65	4.74	1.76	2.34

sample 24, ET 118

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.7	1.68	0.93	1.01	0.56	0.22	0.23
$C_d \times F_d$	48.5	7.63	4.20	4.58	2.55	0.98	1.06
C_p	18.3	4.25	0.75	2.98	1.41	0.43	0.81
C_t	66.82	11.9	4.95	7.57	3.96	1.41	1.87
$M\%C_t$	67.8	12.1	5.03	7.68	4.03	1.43	1.90
$C_t \times F_f$	90.4	16.1	6.70	10.2	5.36	1.91	2.53

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	9	13	18	25
ET	46	70	94	118
starch	225.4	7.66	2.38	2.46
S.D.	54.2	1.55	0.16	0.29
gelatin	1.72	3.46	2.72	2.68
S.D.	0.18	0.07	0.32	0.34
cellulose	0.34	0.88	1.52	1.48
S.D.	0.14	0.18	0.36	0.39

d) Total counts (bacteria X 10^8, protozoa X 10^4; means of 5 counts, not adjusted for F_f)

sample	3	7	12	17	23
ET	21	45	69	93	117
bacteria	1.76	1.16	1.16	1.36	1.40
S.D.	0.64	0.28	0.66	0.59	0.35
protozoa	5.06	3.04	1.28	1.44	0.58
S.D.	0.94	0.32	0.38	0.32	0.14

iv Wilted/formic acid (Run 18)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	2	7	12	19	24
ET	21	45	69	93	116
mg/l	257	234	213	208	212
S.D.	17.3	5.89	4.37	6.52	7.11

b) VFA (mM/l)

sample 4, ET 23

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	16.7	3.56	0.26	1.79	0.55	0.49	0.09
$C_d \times F_d$	84.4	17.9	1.30	9.04	2.76	2.64	0.47
C_p	38.5	10.3	0.81	7.32	1.65	1.00	0.30
C_t	123	28.2	2.11	16.4	4.41	3.46	0.78
$M\%C_t$	80.7	18.5	1.39	10.7	2.89	2.27	0.51
$C_t \times F_f$	177	40.7	3.05	23.6	6.36	4.99	1.12

sample 9, ET 47

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.4	1.90	0.11	1.76	0.38	0.16	0.23
$C_d \times F_d$	52.5	9.58	0.56	8.88	1.91	0.82	1.18
C_p	17.7	3.71	0.23	3.73	1.02	0.28	0.71
C_t	70.2	13.2	0.79	12.6	2.93	1.12	1.89
$M\%C_t$	68.3	12.9	0.77	12.3	2.85	1.08	1.83
$C_t \times F_f$	101	19.1	1.14	18.2	4.22	1.61	2.73

sample 14, ET 71

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.1	1.85	0.07	1.53	0.25	0.16	0.37
$C_d \times F_d$	56.0	9.33	0.37	7.72	1.27	0.82	1.88
C_p	24.0	3.92	0.41	3.11	0.63	0.36	1.12
C_t	80.1	13.2	0.78	10.8	1.90	1.18	3.00
$M\%C_t$	72.1	11.9	0.64	9.75	1.71	1.06	2.70
$C_t \times F_f$	115	19.1	1.13	15.6	2.74	1.70	4.33

sample 21, ET 95

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.6	1.99	0.07	1.62	0.17	0.16	0.47
$C_d \times F_d$	53.2	10.0	0.37	8.18	0.85	0.82	2.36
C_p	21.9	4.13	0.47	3.85	0.39	0.36	1.32
C_t	75.2	14.2	0.84	12.0	1.24	1.18	3.67
$M\%C_t$	69.4	13.1	0.77	9.00	1.15	1.08	3.39
$C_t \times F_f$	108	20.5	1.21	17.4	1.79	1.70	5.29

sample 26, ET 117

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.76	1.90	0.07	1.58	0.13	0.22	0.47
$C_d \times F_d$	44.2	9.58	0.37	7.95	0.64	1.09	2.36
C_p	25.4	4.13	0.41	3.97	0.39	0.35	1.42
C_t	79.6	13.7	0.78	11.9	1.03	1.45	3.78
$M\%C_t$	70.9	12.2	0.69	10.6	0.92	1.29	3.36
$C_t \times F_f$	114	19.8	1.12	17.2	1.48	2.09	5.45

c) Viable counts (X 10^5, means of 3 titres, not adjusted for F_f)

sample	9	14	21	26
ET	47	71	95	117
starch	10.0	17.12	20.4	23.4
S.D.	0.60	2.00	0.14	4.00
gelatin	12.46	4.88	5.80	5.72
S.D.	0.80	0.52	0.79	0.55

d) Total counts (bacteria X 10^8, protozoa X 10^4, means of 5 counts, not adjusted for F_f)

sample	3	8	13	20	25
ET	22	46	70	94	116
bacteria	1.88	2.62	2.86	1.36	2.04
S.D.	0.22	0.55	0.55	0.20	0.52
protozoa	1.25	2.11	1.25	1.73	1.73
S.D.	0.13	0.82	0.41	0.33	0.38

3.B.3 Group 3 silages.

i Control silage (Run 24)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	1	5	10	15	20	24
ET	21	45	140	168	189	213
mg/l	400	271	226	240	238	238
S.D.	9.29	2.65	6.06	1.84	2.94	1.30

sample 21, ET 189

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.34	2.19	0.14	1.85	0.29	0.18	0.22
$C_d \times F_d$	30.5	8.04	0.51	6.78	1.07	0.65	0.81
C_p	24.4	4.92	0.77	3.66	0.98	0.39	0.04
C_t	54.9	12.9	1.28	10.4	2.06	1.05	0.85
$M\%C_t$	65.7	15.5	1.54	12.5	2.47	1.26	1.01
$C_t \times F_f$	74.3	17.6	1.74	14.1	2.79	1.43	1.15

sample 25, ET 213

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.37	2.20	1.14	2.07	0.37	0.25	0.17
$C_d \times F_d$	34.3	8.06	0.51	7.60	1.34	0.90	0.62
C_p	25.8	4.91	0.74	3.87	0.82	0.38	0.28
C_t	60.1	12.9	1.25	11.5	2.17	1.28	0.89
$M\%C_t$	66.7	14.4	1.38	12.7	2.40	1.42	0.99
$C_t \times F_f$	81.5	17.5	1.69	15.5	2.93	1.73	1.21

c) Viable counts (X 10^5 means of 3 tubes; not adjusted for F_f)

sample	9	14	19	28
ET	48	145	167	215
starch	10.6	10.0	10.6	11.1
S.D.	1.40	0.80	1.22	0.90
gelatin	1.31	1.51	1.64	1.69
S.D.	0.26	0.23	0.15	0.22
cellulose	4.46	0.99	1.02	1.12
S.D.	0.50	0.07	0.16	0.15

d) Total counts (bacteria X 10^8, protozoa X 10^4; means of 5 counts, not adjusted for F_f)

sample	3	7	12	17	22	26
ET	22	46	143	166	190	214
bacteria	2.54	2.58	3.10	1.60	2.12	2.94
S.D.	0.58	0.59	0.24	0.21	0.64	0.56
protozoa	5.34	3.87	2.30	1.86	1.15	1.41
S.D.	0.73	0.75	0.68	0.37	0.56	0.21

ii Formaldehyde/formic acid silage (Run 25)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	1	6	11	16	28
ET	44	68	92	117	141
mg/l	208	129	129	131	128
S.D.	00.0	0.21	0.21	0.13	0.21

b) VFA (mM/l)

sample 2, ET 44

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.68	1.41	0.89	1.26	0.37	0.25	0.09
$C_d \times F_d$	47.2	6.89	0.43	6.13	1.79	1.24	0.43
C_p	28.8	5.91	0.33	4.28	1.21	0.56	0.39
C_t	75.5	12.8	0.77	10.4	2.99	1.79	0.82
$M\%C_t$	71.84	12.2	0.73	9.91	2.84	1.71	0.78
$C_t \times F_f$	119	20.2	1.21	16.4	4.73	2.84	0.45

sample 8, ET 68

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.19	1.15	0.07	1.07	0.23	0.18	0.09
$C_d \times F_d$	39.9	5.59	0.34	5.25	1.14	0.88	0.46
C_p	16.5	3.69	0.23	2.57	0.69	0.29	0.19
C_t	56.4	9.28	0.58	7.82	2.84	1.17	1.65
$M\%C_t$	70.7	11.6	0.73	9.80	2.99	1.47	2.07
$C_t \times F_f$	89.0	14.6	0.92	12.3	4.48	1.85	2.61

sample 12, ET 92

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.07	1.19	0.89	1.07	0.24	0.21	0.13
$C_d \times F_d$	39.3	5.81	0.43	5.25	1.19	1.03	0.65
C_p	18.7	3.58	0.40	2.96	0.57	0.47	0.34
C_t	58.0	9.40	0.83	8.10	1.75	1.49	0.99
$M\%C_t$	71.98	11.6	1.03	10.1	2.17	1.86	1.23
$C_t \times F_f$	91.6	14.8	1.31	12.8	2.77	2.36	1.57

sample 17, ET 117

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.6	1.52	0.11	1.33	0.34	0.28	0.13
$C_d \times F_d$	51.8	7.40	0.52	6.49	1.64	1.37	0.63
C_p	17.7	3.46	0.54	2.90	0.63	0.35	0.32
C_t	69.5	10.8	1.05	9.39	2.37	1.72	0.97
$M\%C_t$	72.5	11.0	1.09	9.80	2.47	1.79	0.98
$C_t \times F_f$	109	17.1	1.66	14.8	3.74	1.85	1.49

sample 21, ET 141

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.19	1.48	0.08	1.33	0.27	0.22	0.11
$C_d \times F_d$	44.8	7.22	0.34	6.49	1.31	1.05	0.53
C_p	18.2	3.52	0.50	2.93	0.58	0.42	0.33
C_t	63.0	10.7	0.84	9.42	1.89	1.48	0.85
$M\%C_t$	71.6	12.2	0.51	10.7	2.16	1.68	0.62
$C_t \times F_f$	99.4	16.9	1.33	14.9	2.98	2.33	1.35

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	5	10	15	20
ET	46	70	94	142
starch	1.15	1.63	1.96	1.98
S.D.	0.06	0.09	0.30	0.17
gelatin	1.11	1.18	1.22	1.23
S.D.	0.15	0.05	0.14	0.18
cellulose	1.05	1.17	1.29	1.18
S.D.	0.15	0.11	0.02	0.12

d) Total counts (bacteria X 10^8, protozoa X 10^4; means of 5 counts, not adjusted for F_f)

sample	4	9	14	20
ET	45	69	93	142
bacteria	1.54	1.08	1.56	1.58
S.D.	0.20	0.49	0.29	0.52
protozoa	4.67	2.88	2.40	1.95
S.D.	1.41	0.51	0.39	0.50

iii Formaldehyde/H_2SO_4 silage (Run 19)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	2	6	10	16	21
ET	21	45	69	93	123
mg/l	296	297	301	297	298
S.D.	0.55	0.63	0.01	0.25	0.38

b) VFA (mM/l)

sample 4, ET 23

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	12.1	1.99	0.23	1.63	0.49	0.27	0.11
$C_d \times F_d$	53.5	8.84	1.02	7.21	2.16	1.00	0.49
C_p	36.4	5.74	0.74	5.58	1.47	0.65	0.41
C_t	89.9	14.6	1.76	12.8	3.63	1.66	0.91
$M\%C_t$	71.8	11.6	1.41	14.0	2.89	1.33	0.72
$C_t \times F_f$	127	20.6	2.49	18.1	5.14	2.35	1.28

sample 9, ET 48

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	16.7	3.32	0.18	2.57	0.65	0.36	0.19
$C_d \times F_d$	73.9	14.7	0.78	9.98	2.90	1.60	0.87
C_p	56.3	12.7	1.30	6.59	2.35	0.91	0.68
C_t	130	27.4	2.09	16.5	5.24	2.51	1.55
$M\%C_t$	70.2	14.7	1.12	8.93	2.83	1.35	0.83
$C_t \times F_f$	184	38.7	2.95	23.5	7.44	3.55	2.19

sample 12, ET 71

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	15.5	3.57	0.18	2.20	0.43	0.21	0.16
$C_d \times F_d$	68.8	15.8	0.78	9.76	1.91	0.94	0.69
C_p	44.8	9.82	1.08	5.63	1.77	0.93	0.41
C_t	113	25.6	1.87	15.4	3.69	1.87	1.10
$M\%C_t$	69.6	15.7	1.15	9.43	2.26	1.15	0.68
$C_t \times F_f$	160	36.3	2.65	21.7	5.22	2.65	1.57

sample 18, ET 95

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	15.8	3.28	0.18	2.19	0.66	0.36	0.16
$C_d \times F_d$	69.8	14.5	0.78	9.68	2.90	1.60	0.69
C_p	50.5	11.3	1.23	6.33	1.98	0.94	0.54
C_t	120	25.8	2.01	16.0	4.89	1.54	1.24
$M\%C_t$	70.0	15.0	1.17	9.32	2.84	0.89	0.72
$C_t \times F_f$	170	36.6	2.86	22.7	6.92	2.18	1.76

sample 23, ET 124

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	17.0	4.01	0.15	2.35	0.56	0.40	0.20
$C_d \times F_d$	75.3	17.8	0.67	10.3	2.49	1.77	0.88
C_p	54.8	13.6	1.08	6.84	1.90	0.92	0.49
C_t	130	31.4	1.75	17.2	4.39	2.69	1.38
$M\%C_t$	68.7	16.6	0.93	9.08	2.33	1.42	0.73
$C_t \times F_f$	184	44.5	2.48	24.3	6.14	3.81	1.96

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	9	13	19	24
ET	48	71	95	124
starch	37.9	27.3	25.1	26.2
S.D.	3.78	2.81	2.80	3.2
gelatin	15.3	11.7	10.7	9.0
S.D.	1.81	1.10	1.51	1.00
cellulose	21.6	16.3	17.7	16.3
S.D.	2.16	0.99	1.72	1.70

d) Total counts (bacteria X 10^8, protozoa X 10^4; means of 5 counts, not adjusted for F_f)

sample	3	7	11	17	22
ET	22	46	70	94	124
bacteria	3.90	2.34	2.50	1.88	1.62
S.D.	0.52	0.57	0.78	0.39	0.34
protozoa	4.96	3.65	1.50	1.60	0.58
S.D.	0.72	0.51	0.50	0.66	0.33

iv Formaldehyde-only silage (Run 20)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	2	6	11	15	20
ET	21	45	69	93	140
mg/l	312	239	231	235	234
S.D.	0.92	0.96	1.05	0.21	0.38

b) VFA (mM/l)

sample 4, ET 23

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	7.22	1.02	0.07	0.97	0.21	0.14	0.6
$C_d \times F_d$	32.7	4.61	0.32	4.40	0.95	0.66	0.27
C_p	44.8	7.92	0.97	6.87	1.65	1.03	0.38
C_t	77.5	12.5	1.29	11.3	2.60	1.68	0.65
$M\%C_t$	72.0	11.6	1.21	10.5	2.42	1.56	0.60
$C_t \times F_f$	112	18.1	1.88	16.3	3.78	2.45	0.94

sample 9, ET 48

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.7	1.99	0.09	1.70	0.46	0.36	0.19
$C_d \times F_d$	53.0	9.03	0.40	7.73	2.08	1.64	0.89
C_p	36.8	6.38	0.65	5.72	1.47	0.75	0.29
C_t	89.9	15.4	0.95	13.4	3.54	2.39	1.18
$M\%C_t$	70.9	12.1	0.75	10.6	2.79	1.88	0.93
$C_t \times F_f$	130	22.4	1.38	19.5	5.14	3.47	1.72

sample 17, ET 71

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.6	1.54	0.09	1.45	0.47	0.33	0.20
$C_d \times F_d$	52.5	7.00	0.41	6.57	2.12	1.47	0.93
C_p	29.1	5.13	0.58	4.78	0.94	0.65	0.39
C_t	81.6	12.1	0.99	11.3	3.07	2.13	1.32
$M\%C_t$	72.5	10.7	0.88	10.1	2.7	1.89	1.17
$C_t \times F_f$	118	17.6	1.44	16.5	4.45	3.09	1.92

sample 20, ET 95

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.1	1.96	0.09	1.87	0.40	0.32	0.23
$C_d \times F_d$	50.3	8.89	0.40	8.49	1.82	1.48	1.07
C_p	32.0	6.35	0.59	4.59	1.31	0.58	0.36
C_t	82.3	15.2	1.01	13.1	3.14	2.06	1.43
$M\%C_t$	69.6	12.8	0.85	11.1	2.65	1.74	1.21
$C_t \times F_f$	119	22.1	1.47	18.9	4.56	2.99	2.08

sample 24, ET 141

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.5	1.91	0.08	1.75	0.35	0.31	0.21
$C_d \times F_d$	52.2	8.63	0.39	7.93	1.39	1.39	0.95
C_p	33.0	6.30	0.63	4.89	1.37	0.57	0.39
C_t	85.2	14.9	1.01	12.8	2.77	1.97	1.04
$M\%C_t$	71.1	12.5	0.85	12.7	2.31	1.65	0.87
$C_t \times F_f$	123	21.7	1.47	18.6	4.02	2.86	1.51

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	8	13	17	24
ET	48	71	95	142
starch	18.3	14.3	12.6	12.3
S.D.	1.51	1.75	1.20	1.67
gelatin	1.88	1.62	1.66	1.69
S.D.	0.16	0.13	0.17	0.11
cellulose	0.63	5.33	5.53	5.07
S.D.	0.13	0.64	0.99	0.31

d) Total counts (bacteria X 10^8, protozoa X 10^4; means of 5 counts, not adjusted for F_f)

sample	3	7	12	16	21
ET	21	46	70	94	141
bacteria	1.04	0.86	0.78	1.68	1.56
S.D.	0.39	0.31	0.24	0.42	0.40
protozoa	4.67	4.13	2.02	2.30	2.21
S.D.	1.29	1.01	0.49	0.29	0.48

3.C In vitro infusion experiments.

3.C.1 Formate infusion (Run 26)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	1	5	10	15	20	25	30	35
period	⌐— A —¬		1	2	3	4	5	6
mg/l	82.8	85.4	85.4	82.4	90.0	103	109	114
S.D.	1.30	3.15	2.77	1.85	1.18	1.47	2.61	2.14

b) VFA (mM/l)

sample 2, period A1

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.1	2.16	0.08	1.56	0.15	0.17	0.10
$C_d \times F_d$	26.5	5.14	0.19	3.71	0.36	0.40	0.24
C_p	37.7	7.88	0.69	4.96	1.18	0.97	0.96
C_t	64.2	13.0	0.88	8.67	1.54	1.37	1.20
$M\%C_t$	70.6	14.3	0.97	9.54	1.69	1.51	1.32
$C_t \times F_f$	86.7	17.6	1.19	11.7	2.08	1.85	1.62

sample 6, period A2

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.8	2.09	0.13	1.47	0.23	0.24	0.21
$C_d \times F_d$	25.7	4.97	0.31	3.49	0.55	0.57	0.50
C_p	36.2	8.71	0.64	5.71	1.22	1.00	0.96
C_t	61.9	13.7	0.95	9.20	1.77	1.57	1.46
$M\%C_t$	68.4	15.1	1.05	10.1	1.95	1.73	1.61
$C_t \times F_f$	83.6	18.5	1.28	12.4	2.39	2.12	1.97

sample 10, period 1

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.3	2.16	0.13	1.53	0.20	0.24	0.24
$C_d \times F_d$	27.6	5.26	0.31	3.73	0.49	0.58	0.58
C_p	31.6	5.80	0.54	5.48	0.92	0.86	1.07
C_t	59.3	11.6	0.85	9.21	1.41	1.44	1.65
$M\%C_t$	69.4	13.6	0.99	10.8	1.65	1.68	1.93
$C_t \times F_f$	77.9	15.2	1.12	12.1	1.85	1.89	2.17

sample 16, period 2

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.58	1.48	0.08	1.06	0.20	0.17	0.17
$C_d \times F_d$	20.3	3.50	0.19	2.50	0.47	0.40	0.40
C_p	25.9	3.94	0.69	5.34	0.92	0.93	1.18
C_t	46.3	7.44	0.88	7.84	1.39	1.33	1.58
$M\%C_t$	69.3	11.4	1.32	11.7	2.08	1.99	2.37
$C_t \times F_f$	62.7	10.1	1.19	10.6	1.88	1.80	2.14

sample 21, period 3

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	8.23	1.72	0.10	0.94	0.15	0.17	0.14
$C_d \times F_d$	19.3	4.03	0.23	2.20	0.35	0.40	0.33
C_p	23.3	4.56	0.59	6.22	0.88	0.86	1.28
C_t	42.6	8.59	0.82	8.42	1.23	1.26	1.61
$M\%C_t$	66.0	13.3	1.27	13.0	1.90	1.95	2.49
$C_t \times F_f$	58.5	11.8	1.24	11.5	1.69	1.73	2.21

sample 26, period 4

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	7.72	1.36	0.08	0.88	0.15	0.21	0.21
$C_d \times F_d$	17.1	3.02	0.18	1.95	0.33	0.46	0.46
C_p	21.5	3.42	0.69	5.93	0.88	0.93	1.12
C_t	38.6	6.44	0.87	7.88	1.21	1.39	1.58
$M\%C_t$	66.7	11.1	1.50	13.6	2.09	2.40	2.73
$C_t \times F_f$	55.7	9.30	1.26	11.3	1.75	2.01	2.28

sample 31, period 5

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	6.85	0.74	0.08	1.03	0.18	0.17	0.21
$C_d \times F_d$	14.6	1.58	0.17	2.20	0.38	0.36	0.45
C_p	17.7	2.90	0.43	5.56	0.53	0.72	0.96
C_t	32.3	4.48	0.60	7.76	0.91	1.08	1.41
$M\%C_t$	66.6	9.22	1.23	15.9	1.87	2.22	2.90
$C_t \times F_f$	48.5	6.72	0.90	11.6	1.36	1.62	2.11

sample 36, period 6

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	3.88	0.92	0.08	0.53	0.18	0.14	0.17
$C_d \times F_d$	7.57	1.79	0.16	1.03	0.35	0.27	0.33
C_p	12.2	2.49	0.54	3.29	0.49	0.64	1.07
C_t	19.8	4.28	0.70	4.32	0.84	0.91	1.40
$M\%C_t$	61.4	13.3	2.17	13.4	2.60	2.82	4.34
$C_t \times F_f$	32.5	7.02	1.15	7.09	1.38	1.49	2.30

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	7	14	19	24	29	34	39
period	A2	1	2	3	4	5	6
starch	1.23	1.12	0.92	0.97	2.03	1.03	$<10^4$
S.D.	0.11	0.06	0.03	0.02	0.16	0.18	-
gelatin	2.26	12.9	1.33	1.45	19.7	1.16	$<10^4$
S.D.	0.03	0.23	0.59	0.67	0.14	0.11	-
cellulose	1.03	11.2	8.5	10.1	16.1	15.1	$<10^4$
S.D.	0.07	0.07	0.11	0.06	0.16	0.02	-

d) Total counts (bacteria X 10^8, protozoa X 10^4; means of 5 counts, not adjusted for F_f)

sample	9	13	17	22	27	32	37
period	A	1	2	3	4	5	6
bacteria	1.08	1.12	1.48	1.04	0.72	0.66	0.60
S.D.	0.30	0.13	0.44	0.40	0.13	0.35	0.20
protozoa	1.22	1.63	1.47	0.69	1.12	1.09	1.34
S.D.	0.27	0.55	0.26	0.24	0.96	0.31	0.70

3.C.2 Acetate infusion (Run 27)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	1	5	10	15	20	25	30
period	⌐A¬		1	2	3	4	5
mg/l	68.9	101	104	109	99.7	120	137
S.D.	2.27	0.92	4.62	0.92	1.89	7.95	3.61

b) VFA (mM/l)

sample 2, period A1

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.4	2.34	0.14	1.77	0.29	0.21	0.14
$C_d \times F_d$	23.3	5.24	0.31	3.96	0.65	0.47	0.31
C_p	42.2	9.33	0.64	5.85	1.26	0.93	0.96
C_t	65.5	14.6	0.95	9.81	1.91	1.40	1.27
$M\%C_t$	68.7	15.3	0.99	10.3	2.00	1.47	1.33
$C_t \times F_f$	86.5	19.2	1.25	12.9	2.52	1.85	1.68

sample 6, period A2

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.0	2.03	0.14	1.68	0.23	0.21	0.10
$C_d \times F_d$	22.4	4.54	0.31	3.76	0.51	0.47	0.22
C_p	42.9	9.02	0.54	5.48	1.14	0.79	0.80
C_t	65.4	13.5	0.85	9.24	1.65	1.26	1.02
$M\%C_t$	70.3	14.6	0.91	9.94	1.77	1.35	1.10
$C_t \times F_f$	86.4	17.9	1.22	12.2	2.18	1.66	1.35

sample 11, period 1

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.61	1.66	0.10	1.53	0.26	0.14	0.14
$C_d \times F_d$	21.0	3.63	0.22	3.35	0.57	0.31	0.31
C_p	38.8	8.08	0.43	5.56	1.03	0.54	0.80
C_t	60.2	11.7	0.65	8.91	1.60	0.85	1.11
$M\%C_t$	70.8	13.8	0.76	10.5	1.88	1.00	1.31
$C_t \times F_f$	81.7	15.9	0.88	12.1	2.17	1.56	1.51

sample 16, period 2

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	13.1	1.20	0.08	1.37	0.15	0.14	0.21
$C_d \times F_d$	28.6	2.63	0.17	3.00	0.33	0.31	0.46
C_p	46.3	5.80	0.35	5.63	0.69	0.39	0.86
C_t	74.9	8.43	0.52	8.63	1.02	0.70	1.32
$M\%C_t$	78.4	8.82	0.54	9.03	1.07	0.73	1.38
$C_t \times F_f$	106	11.4	0.71	11.7	1.39	0.95	1.79

sample 21, period 3

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	22.1	1.05	0.33	1.59	0.23	0.17	0.31
$C_d \times F_d$	46.1	2.19	0.69	3.32	0.48	0.35	0.65
C_p	67.1	2.28	0.16	2.26	0.15	0.14	0.37
C_t	113	4.47	0.85	5.58	0.63	0.49	1.02
$M\%C_t$	89.6	3.54	0.67	4.42	0.50	0.39	0.81
$C_t \times F_f$	161	6.38	1.21	7.96	0.90	0.70	1.45

sample 26, period 4

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	41.8	0.86	0.41	1.59	0.20	0.14	0.34
$C_d \times F_d$	84.5	1.74	0.83	3.21	0.40	0.28	0.69
C_p	112	2.49	0.19	3.22	0.38	0.21	0.48
C_t	196	4.23	1.02	6.43	0.78	0.49	1.17
$M\%C_t$	93.3	2.00	0.48	3.05	0.37	0.23	0.55
$C_t \times F_f$	285	6.13	1.48	9.32	1.13	0.71	1.70

sample 31, period 5

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	102	0.99	0.50	1.29	0.20	0.14	0.34
$C_d \times F_d$	-	1.83	0.92	2.38	0.37	0.29	0.63
C_p	145	2.07	0.16	3.63	0.46	0.36	0.64
C_t	-	3.90	1.08	6.01	0.83	0.65	1.27
$M\%C_t$	-	-	-	-	-	-	-
$C_t \times F_f$	-	6.28	1.74	9.68	1.34	1.46	2.04

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	9	14	19	24	29	34
period	A	1	2	3	4	5
starch	2.28	1.26	1.29	9.13	94	$> 10^6$
S.D.	0.22	0.24	0.10	0.50	4.16	-
gelatin	1.64	1.50	0.73	11.2	89.3	$> 10^6$
S.D.	0.18	0.45	0.05	0.58	14.4	-
cellulose	0.95	0.87	1.23	0.60	0.64	$< 10^4$
S.D.	0.24	0.28	0.52	0.47	0.09	-

d) Total counts (bacteria X 10^8, protozoa X 10^4, means of 5 counts, not adjusted for F_f)

sample	4	8	13	18	23	28	33
period	A1	A2	1	2	3	4	5
bacteria	1.54	1.18	1.50	0.72	0.92	0.62	0.65
S.D.	0.36	0.40	0.32	0.25	0.08	0.16	0.12
protozoa	1.09	1.06	0.93	0.70	0.74	0.86	0.11
S.D.	0.20	0.36	0.03	0.03	0.01	0.05	0.03

3.C.3 Propionate infusion (Run 28)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	1	6	11	16	21	26	31
period	⌐—A—¬		1	2	3	4	5
mg/l	78.6	89.5	90.0	86.2	96.3	113	133
S.D.	1.68	2.81	1.64	1.59	2.77	0.63	1.85

b) VFA (mM/l)

sample 2, period A1

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.4	2.37	0.10	1.24	0.23	0.21	0.14
$C_d \times F_d$	28.6	5.93	0.25	3.10	0.57	0.52	0.35
C_p	39.9	8.71	0.46	4.89	1.07	0.82	0.59
C_t	68.6	14.6	0.71	9.03	1.64	1.34	0.94
$M\%C_t$	70.8	15.1	0.73	9.32	1.69	1.38	0.97
$C_t \times F_f$	89.8	19.1	0.93	11.8	2.15	1.75	1.23

sample 7, period A2

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	11.1	2.53	0.15	1.13	0.12	0.21	0.21
$C_d \times F_d$	27.8	6.33	0.37	2.83	0.30	0.52	0.52
C_p	42.6	8.19	0.38	5.19	0.69	0.86	0.54
C_t	70.4	14.5	0.75	8.02	0.99	1.38	1.06
$M\%C_t$	72.5	14.9	0.77	8.25	1.02	1.42	1.09
$C_t \times F_f$	92.2	19.0	0.98	10.5	1.29	1.81	1.38

sample 12, period 1

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.6	8.06	0.29	0.98	0.12	0.04	0.07
$C_d \times F_d$	26.8	20.3	0.37	2.46	0.30	0.08	0.18
C_p	37.9	10.3	0.35	3.62	0.69	0.10	0.22
C_t	64.8	30.7	0.72	6.08	0.99	0.18	0.40
$M\%C_t$	62.5	29.6	0.69	5.86	0.96	0.18	0.40
$C_t \times F_f$	83.9	39.7	0.93	7.88	1.24	0.23	0.52

sample 17, period 2

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	12.4	12.7	-	0.94	0.23	0.41	-
$C_d \times F_d$	32.3	33.6	-	2.44	0.60	1.06	-
C_p	16.2	17.1	0.78	2.45	0.43	1.24	0.21
C_t	48.5	50.1	0.78	4.89	1.03	2.30	0.21
$M\%C_t$	44.9	46.5	0.72	4.54	0.96	2.13	0.19
$C_t \times F_f$	61.0	63.1	0.98	6.16	1.30	2.89	0.26

sample 22, period 3

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.7	49.6	-	0.62	0.18	0.69	-
$C_d \times F_d$	26.6	123	-	1.54	0.45	1.71	-
C_p	15.6	66.7	0.75	2.52	0.76	2.25	-
C_t	42.2	82.1	0.75	4.06	1.21	3.96	-
$M\%C_t$	31.4	61.1	0.31	3.02	0.50	1.64	-
$C_t \times F_f$	55.7	108	0.99	5.36	1.59	5.22	-

sample 27, period 4

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	6.64	≈ 65	-	0.59	0.17	1.04	-
$C_d \times F_d$	15.9	≈ 155	-	1.41	0.42	2.49	-
C_p	10.5	118	1.39	2.15	0.53	3.07	-
C_t	26.4	≈ 275	1.39	3.56	0.95	5.56	-
$M\%C_t$	≈ 15	≈ 90	-	≈ 2	-	-	-
$C_t \times F_f$	36.1	> 300	1.89	4.85	1.29	7.58	-

sample 32, period 5

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	4.29	-	-	0.47	0.18	1.11	-
$C_d \times F_d$	9.38	-	1.03	1.03	0.39	2.43	-
C_p	3.56	>300	0.69	0.73	0.76	1.48	-
C_t	12.9	-	1.76	1.76	1.05	3.91	-
$M\%C_t$	≈4.5	≈95	-	<1	-	-	-
$C_t \times F_f$	19.3	>400	-	2.63	1.57	5.85	-

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	5	10	15	20	25	30	35
period	⌐—A—¬		1	2	3	4	5
starch	2.74	5.31	318	231	-	439	9.55
S.D.	0.14	0.39	38.6	10.7	-	41.3	2.20
gelatin	1.77	2.28	329	26.2	-	79.3	5.13
S.D.	0.10	0.43	27.2	3.02	-	4.16	1.93
cellulose	0.61	0.86	1.14	0.62	-	6.46	1.26
S.D.	0.14	0.08	0.07	0.69	-	1.55	-.58

d) Total counts (bacteria X 10^8, protozoa X 10^4, means of 5 counts, not adjusted for F_f)

sample	3	8	13	18	23	28	34
period	⌐—A—¬		1	2	3	4	5
bacteria	1.48	1.12	1.08	0.98	0.62	0.34	0.20
S.D.	0.54	0.38	0.22	0.31	0.28	0.44	0.07
protozoa	1.15	0.80	0.77	0.91	0.83	0.64	0.54
S.D.	0.28	0.03	0.03	0.04	0.03	0.04	0.33

3.C.4 Formaldehyde infusion (Run 29)

a) NH_3-N (means of 5 titres, not adjusted for F_f)

sample	1	3	8	13	18
period	⌐—A—¬		1	2	3
mg/l	87.1	89.6	60.9	13.9	3.78
S.D.	1.68	1.68	2.10	0.88	0.38

b) VFA (mM/l)

sample 2, period A1

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	9.81	2.28	0.15	1.09	0.23	0.24	0.14
$C_d \times F_d$	22.9	5.34	0.35	2.55	0.54	0.56	0.33
C_p	43.7	9.54	0.54	5.48	1.07	0.93	0.86
C_t	66.7	14.9	0.89	8.03	1.61	1.49	1.19
$M\%C_t$	70.4	15.7	0.94	8.47	1.70	1.57	1.25
$C_t \times F_f$	88.5	19.7	1.18	10.7	2.14	1.98	1.58

sample 4, period A2

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	10.3	2.23	0.20	1.18	0.26	0.28	0.07
$C_d \times F_d$	24.2	5.22	0.47	2.76	0.61	0.65	0.16
C_p	45.2	10.4	0.38	4.52	0.84	0.86	0.91
C_t	69.4	15.6	0.85	7.28	1.45	1.51	1.07
$M\%C_t$	71.4	16.0	0.87	7.49	1.49	1.18	1.10
$C_t \times F_f$	92.1	20.7	1.13	9.67	1.92	2.00	1.42

sample 9, period 1

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	3.17	0.31	0.23	0.24	0.18	-	-
$C_d \times F_d$	7.86	0.77	0.57	0.60	0.45	-	-
C_p	18.8	5.18	0.54	2.00	0.49	0.36	-
C_t	26.7	5.95	1.11	2.60	0.94	0.36	-
$M\%C_t$	70.9	15.8	2.95	6.90	2.49	0.96	-
$C_t \times F_f$	33.6	7.50	1.40	3.28	1.18	0.45	-

sample 14, period 2

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	1.53	0.18	0.30	0.12	-	-	-
$C_d \times F_d$	3.79	0.45	0.74	-.30	-	-	-
C_p	12.4	1.97	0.86	0.96	0.15	0.11	-
C_t	16.2	2.42	1.60	1.26	0.15	0.11	-
$M\%C_t$	74.8	11.1	2.46	7.35	0.23	0.16	-
$C_t \times F_f$	20.4	3.05	2.02	1.59	0.19	0.14	-

sample 19, period 3

	C_2	C_3	iC_4	C_4	iC_5	C_5	C_6
C_d	0.82	-	0.23	0.12	-	-	-
$C_d \times F_d$	2.09	-	0.59	0.31	-	-	-
C_p	8.85	0.41	0.75	0.52	-	-	-
C_t	10.9	0.41	1.34	0.83	-	-	-
$M\%C_t$	80.9	3.0	9.90	6.13	-	-	-
$C_t \times F_f$	13.5	0.50	1.65	1.02	-	-	-

c) Viable counts (X 10^5, means of 3 tubes, not adjusted for F_f)

sample	7	12	17	22
period	A2	1	2	3[1]
starch	61.3	67.3	1.55	0.10
S.D.	10.0	14.7	0.15	-
gelatin	23.9	15.8	72.0	0.27
S.D.	3.38	2.42	11.3	-
cellulose	17.1	1.37	0.25	0.25
S.D.	0.90	0.13	0.03	-

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[1] Counts per tube in period 3 too low for statistical accuracy.

d) Total counts (bacteria X 10^8, protozoa X 10^4, means Of 5 counts, not adjusted for F_f)

sample	5	10	15	20
period	A2	1	2	3
bacteria	1.66	1.48	1.96	0.13
S.D.	0.39	0.34	0.29	0.04
protozoa	0.99	0.96	0.83	0.16
S.D.	0.02	0.04	0.02	0.02

List of papers presented
in support of this thesis.

EWART, J.M. (1973). An inexpensive method of controlled pumping of liquids at low flow rates. Lab. Pract., 22, 575-6.

EWART, J.M. (1974). In vitro rumen studies with silages. Paper presented at 3rd Silage Conference, held at the Edinburgh School of Agriculture, 23-24 September 1974. Unpublished.

EWART, J.M. (1974). Continuous in vitro rumen systems. Proc. Nutr. Soc., 33, 125-133.

EWART, J.M. (1976). In vitro rumen studies with silages. Paper presented at 4th Silage Conference, held at the Grassland Research Institute, Hurley, 22-23 September 1976. Unpublished.

Reprinted from Laboratory Practice, September 1973.

Apparatus and devices

An inexpensive method of controlled pumping of liquids at low flow rates

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Continuous pumping of liquids at low, controlled rates, is required in many types of laboratory equipment such as chemostats and plant growth chambers. It is desirable in most of these applications to have control over a range of delivery rates. Equipment capable of providing such control is generally expensive; the apparatus described here provides accurate and reliable pumping at remarkably low cost.

The basis, of this pumping system is the 'Peripump', a miniature peristaltic pump driven by a synchronous motor *via* a gearbox (Figure 1). By changing the bore of the silicone rubber element tube in the pump and/or the output speed of the gearbox (using interchangeable motor units) a range of flow rates may be obtained from the basic pump unit (Table I).

Drive by synchronous motor endows these pumps with considerable inherent flow accuracy. Fine control of delivery is accomplished by interrupting the electrical supply to the pump motor at regular intervals. This interruption is brought about by an astable (i.e. freerunning) multivibrator circuit which switches the pump on and off *via* a relay.

The circuit (Figure 2) is arranged to provide for variation of both the 'on' time of the pump and the 'off' time. With a maximum time constant of about 20 sec. and a minimum of 1 sec., flow rates from 5 per cent to 95 per cent of the nominal output of the basic pump unit are readily obtained. Accuracy of delivery is better than ± 2.5 per cent of maximum delivery per hour. Greater accuracy, over a narrower delivery range, may be obtained by reducing the value of the 250K variable resistors.

Figure 3 shows complete pump-control units built up as plug-in modules for use in a chemostat type of apparatus. It is necessary to calibrate each control unit/pump/tube-bore combination and plot a graph of delivery obtained against various settings of the control unit variable resistors. A typical calibration graph is shown in Figure 4.

With the 'Peripump' it is necessary to 'run in' new element tubes before calibration and before putting them into service as replacements. Although the prototype equipment uses the 'Peripump' it is obvious that this does not exclude the use of other types of pump.

Total cost of pump and control unit as described is about £10. A parts list for the control unit is given below. Several of these devices have been in use in this laboratory for more than a year and have proved to be completely reliable.

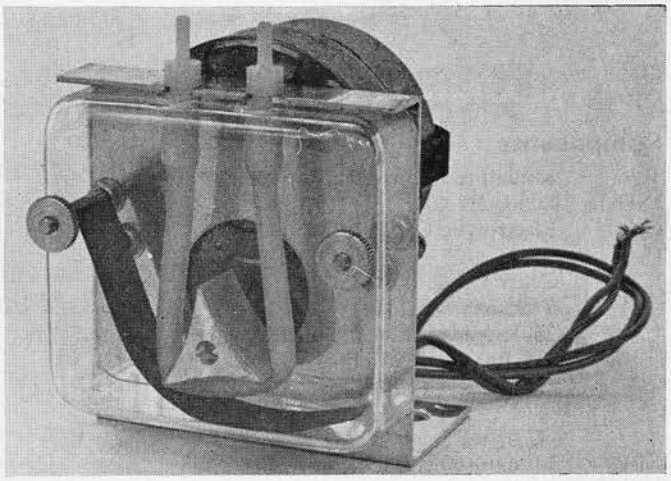

Figure 1.

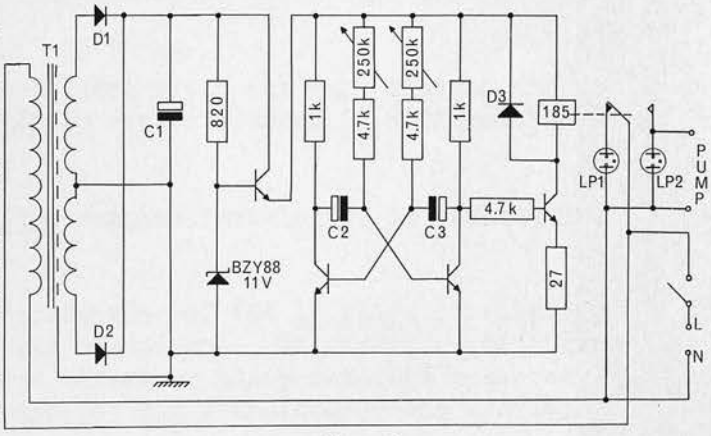

Figure 2.

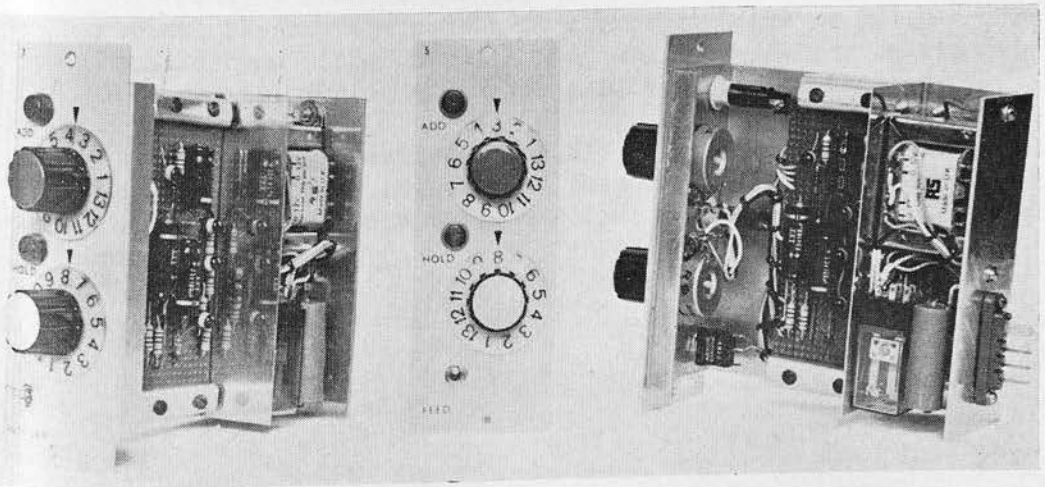

Figure 3. Pump control modules.

Table I

Approximate deliveries (ml hr^{-1}) of basic peripump unit.

Pump Speed (*R.P.M.*)	*Bore of element tube* (*mm*)		
	1.0	2.5	5.0
2	12	30	60
6	30	75	150
10	45	120	240

Components

T.	Miniature Mains Transformer 12V	
$D.D_2D_3$	Diode IN 4001	
C.	Electrolytic Capacitor 47uF 25V	
C_2C_3	„ „ 100uF 25V	
	Zener Diode BZY 88 11V	(1)
	Transistors BC 108	(4)
	250K Midget Linear Potentiometer	(2)
	½W Resistor 27 ohms	(1)
	„ „ 820 „	(1)
	„ „ 1K „	(2)
	„ „ 4.7K „	(3)
LP1	Miniature neon indicator 250V Amber	
LP1	„ „ „ „ Red	
	Relay, 12V DC working S.P.C.O.	(1)

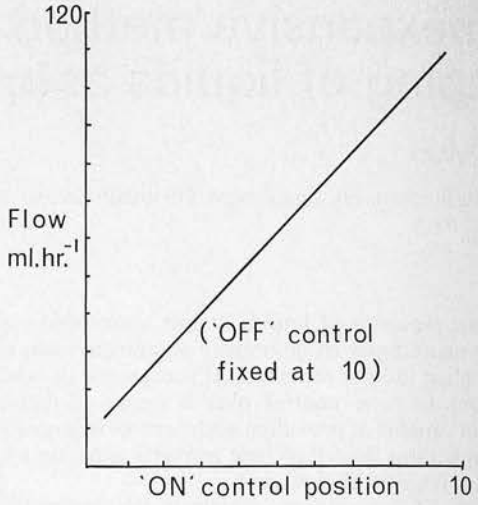

Figure 4. Calibration Graph for 10 R.P.M. Peripump with 5.0 mm bore element tube.

Suppliers

Peripump: Schuco Scientific Ltd., Halliwick Court Place, Woodhouse Road, London, N.12.

Components: R.S. Components Ltd., P.O. Box 427, 13–17 Epworth Street, London, EC2P 2HA.

Acknowledgement

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Third Silage Conference
Edinburgh School of Agriculture
23 - 24 September 1974

In vitro rumen studies with silages

J.M. Ewart
Edinburgh School of Agriculture

The literature contains little information on the effects of silages made with additives upon the rumen microflora. In order to carry out research in this area a new in vitro technique was developed. This technique involved the use of a chemostat type of apparatus arranged to simulate the chemical and physical environment of the rumen.

The equipment was designed to maintain a steady state throughout prolonged culture periods. The microflora supported by these cultural conditions was less diverse than that encountered in vivo but its overall metabolism was similar.

A standardised experimental procedure was adopted, the culture vessel being inoculated with rumen liquor from a group of three fistulated sheep on a fixed diet. The culture was maintained at 39°C, pH 6.5 and a turnover time of 28 hours. The culture attained a steady state after 70-80 hours and was thus maintained for a further 80 hours during the sampling period. Samples were analysed for VFA and NH_3/N. Differential microbial counts on starch, gelatin and cellulose media were also carried out.

Groups of fistulated wether sheep were given the same silages and samples of rumen liquor from these sheep were subjected to differential microbial counts.

The counts obtained with in vitro samples correlated, in most cases, with those of in vivo samples.

The concentration and rate of production of VFA in vitro correlated with the counts of cellulose utilising organisms. In general, additive treated silages did not have a marked effect on the parameters measured compared with the control silages, except when formaldehyde was used as an additive in conjunction with an organic acid.

Silages treated with formaldehyde together with organic acids reduced ruminal NH_3 and the counts of micro-organisms on gelatin-containing medium. Formaldehyde with a mineral acid and formaldehyde on its own was not observed to produce a similar effect.

The concentration of soluble N in the original silage also reflected this trend, being lowest with formaldehyde/organic acid mixtures. Silages treated with these additive mixtures did not deminish VFA production in vitro.

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Continuous in vitro rumen systems

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In vitro rumen experiments have been conducted for at least 40 years, and many research problems have been examined successfully by this approach. The simplest sub-division of in vitro rumen systems is into batch- and continuous-culture types. The batch-culture approach involves the provision of an initial supply only of substrate, which results in a culture in which the concentration of substrates is constantly falling while that of waste products rises. The continuous-culture type of in vitro rumen aims to replenish the substrates and remove waste products over an extended culture period. This is, of course, the situation in vivo and most in vitro systems have employed an intermittent addition of fresh substrate in order to simulate natural conditions as closely as possible. Before evaluating these and other systems it is profitable to consider the nature of the natural rumen as a microbial environment.

Environmental factors in vivo

The rumen is an anaerobic environment which imposes a strictly fermentative metabolism on the microflora. The products of fermentation are relatively reduced compounds, mainly volatile fatty acids (VFA) which the animal almost completely absorbs from the rumen (Gray, 1947; Kiddle, Marshall & Phillipson, 1951). Removal of the VFA is essential because their accumulation causes a diminution of the rate of fermentation (Starks, 1956), presumably by end-product inhibition.

Physical factors are important, as the rumen is a stable microbial environment with temperature virtually constant, mixing at a steady rate, and with transport of materials in and out efficiently conducted by the host. Salivary flow is co-ordinated with food ingestion and this, combined with the fact that undissociated VFA are absorbed more quickly from the rumen than their anions (Masson & Phillipson, 1951; Parthasarathy & Phillipson, 1953), ensures that pH is well controlled under most conditions.

Several workers, Hungate (1966), Hobson (1965), Postgate (1965) and Jannasch (1965) have recognised the analogy of the rumen with the continuous-culture model This implies that a 'realistic' in vitro rumen system must also be continuous.

Having considered the environmental factors encountered in vivo, a set of criteria for an in vitro system may be drawn up. Warner (1956) has established criteria of validity for in vitro rumens which are different from those suggested here as they

were directed toward the microflora itself, not its environment. Thus it is desirable that an in vitro rumen should: (1) be anaerobic; (2) remove products of fermentation; (3) control temperature, pH, mixing and substrate and culture flow; (4) be continuous, if the experimental period exceeds a few hours.

The continuous in vitro rumen

The theory of continuous culture, which was formalized by Monod (1950) and Novick & Szilard (1950), is based on the fact that only one component in the substrate will be limiting microbial growth, and that culture density (the number of organisms present) is controlled by this component. The rate of microbial growth in the culture is dependent only on the rate of flow of substrate and may be controlled, within limits, by adjusting the rate of dilution. A full account of the theory has been given by Powell (1965).

For the theory to apply it is required that the substrate flow rate, and therefore the culture dilution rate, should remain constant and have a value within specified limits. The volume of the culture should also remain invariant. An apparatus enabling these characteristics to be achieved is a chemostat; Herbert, Phipps & Tempest (1965) described the design and instrumentation of the chemostat.

Hungate (1966) has discussed the divergence of the rumen from the continuous culture model, particularly with respect to its non-linear kinetics, and concluded that the value of the continuous culture model lies not so much in its exact applicability as in providing a model for reference.

Many in vitro systems have recognised this divergence from the continuous model and have used periodic 'feeding'. Such systems are generally described as being continuous because the culture has been maintained over an extended period; at least several days. It might be advantageous if cultures were described as continuous only when they exist at a steady state; that is, having the same constitution at all times. Similarly the term 'fermentation patterns' is understood to describe the proportions of fermentation products such as acetic, propionic and butyric acids in the rumen (Hobson, 1972). The fact that these concentrations fluctuate in a periodic manner, in step with nutrient intake, is best illustrated by curve patterns following feeding. It is particularly in the sense that the rumen is 'cyclic' rather than 'steady-state' that the term continuous has a different meaning to the 'ruminologist' than to the microbiologist.

In vitro rumen systems

Table 1 details a number of in vitro systems which have been described. Louw, Williams & Maynard (1949), Adler, Dye, Boggs & Williams (1958), Harbers & Tillman (1962) and Dawson, Ward & Scott (1964) have also described in vitro systems.

Warner (1956) used an apparatus based on that of Louw *et al.* (1949) consisting of a semipermeable sac supported in dialysing solution. The sac contained 50 ml of rumen liquor through which a stream of nitrogen with 5% carbon dioxide was passed. The pH of the culture was adjusted by the addition of buffer solution 'when

Table 1. *Comparison of in vitro rumen systems*

Reference	Type	Mixing	Permeability	pH control
Warner (1956)	B	−	+	−
Davey, Cheeseman & Briggs (1960)	CP	−	+	−
Stewart, Warner & Seeley (1961)	CS	+	−	−
Gray, Weller, Pilgrim & Jones (1962)	BF	+	+	−
Rufener, Nelson & Wolin (1963)	CP	+	−	+
Slyter, Nelson & Wolin (1964)	CFP	+	+	−
Aafjes & Nijhof (1967)	CFB	+	−	+
Czerkawski & Breckenridge (1969)	B	−	−	−

B, batch culture; C, continuous culture; P, periodic feeding; S, steady-state; F, flow-through.
+, featured; −, not featured.

necessary' and the apparatus was maintained at 39° in a water bath. The apparatus reported by Davey, Cheeseman & Briggs (1960) also employed a sac suspended in dialysing solution but the solution was pumped continuously and was not re-used, so that a constant rate of diffusion could be maintained. This was considered to be essential in an apparatus intended for continuous operation. A N_2–CO_2 mixture was bubbled through the culture vessel, which was fitted with sampling ports and a pH electrode system, though no attempt was made to control pH.

Stewart, Warner & Seeley (1961) constructed a steady-state system. The apparatus was a simple chemostat with a mechanical stirrer but without facilities for dialysis.

The use of a double-acting syringe in the apparatus of Gray, Weller, Pilgrim & Jones (1962) allowed the establishment of constant culture volume, since culture material was removed from the vessel at the same rate as saliva was added. This was a semipermeable system with a reciprocating agitator which acted on a wire cage carrying the dialysis membrane. This equipment was employed for batch experiments of a few hours duration.

The impermeable system of Rufener, Nelson & Wolin (1963) was unique in its use of mixed ion-exchange resins for pH control. Agitation of the culture was by external rocking action and, with the culture volume kept constant by withdrawing liquor before adding fresh substrate, cultures were maintained for periods in excess of 200 h with no apparent fall in fermentation rates.

Long-term operation was also achieved with the apparatus of Slyter, Nelson & Wolin (1964), which used a specially constructed fermentation vessel with magnetic stirring. This equipment represented a compromise between the steady-state (chemostat) and periodic (natural rumen) approach. An artificial saliva solution was pumped continuously into the fermentor, providing a constant dilution rate; the substrate was added periodically with a concomitant removal of liquor. This equipment had provision for dialysis.

The continuous-flow, periodic-feed apparatus of Aafjes & Nijhof (1967) had an agitation system designed to simulate the effect of rumen motility. A nylon bag contained the substrate and was attached to a reciprocating plunger running at 5 strokes/min. This was intended to imitate the flow of rumen fluid through the plant material layer as it occurs in the natural rumen. Saliva flow was provided in response to pH drop and a constant volume maintained with a weir overflow.

The apparatus of Czerkawski & Breckenridge (1969) was an impermeable, all-glass, batch-culture system intended for experiments of short duration.

As a stringent test for the artificial rumen, Gray *et al.* (1962) suggested that the molar proportions of the VFA should compare with those of the natural rumen, on the same diet. Table 2 lists the ranges of values observed in those systems cited in Table 1 where such information is available.

Table 2. *Molar proportions of volatile fatty acids (VFA) in in vitro rumen systems*

Reference	Type	Permeability	Molar proportions (mol/100 mol VFA) of: Acetic acid	Propionic acid	Butyric acid
Davey, Cheeseman & Briggs (1960)	CP	+	46–54	19–27	20–24
Stewart, Warner & Seeley (1961)	CS	−	58–63	20–24	11–17
Gray, Weller, Pilgrim & Jones (1962)	BF	+	66–70	16–19	12–15
Rufener, Nelson & Wolin (1963)	CP	−	64–70	17–21	13–15
Slyter, Nelson & Wolin (1964)	CFP	+	66–67	19–20	11–13
Aafjes & Nijhof (1967)	CFP	+	54–69	21–28	9–13
Czerkawski & Breckenridge (1969)	B	−	64–68	19–24	9–12

C, continuous culture; P, periodic feeding; S, steady-state; B, batch culture; F, flow-through.
+, featured; −, not featured.

There appears to be little difference between fermentation patterns in the various types of in vitro rumens. The semipermeable types show similar ranges of proportions to the impermeable systems and no distinctive pattern appears to characterize the batch–culture apparatus compared with the continuous type. Another criterion of validity might be the rate of VFA production, which is higher in the three impermeable systems (Stewart *et al.* 1961; Rufener *et al.* 1963; Czerkawski & Breckenridge, 1969) at 13, 3·7–12 and 13–18 mmol/l per h respectively, than in the semipermeable system of Slyter *et al.* (1964) (4·4 mmol/l per h). The rate reported by Slyter *et al.* was calculated without taking into account the VFA which diffused out via the dialysis system and the actual rate was probably higher than that given. The very high rate reported by Czerkawski & Breckenridge (1969) was for a batch system with high substrate concentrations and, since the figures for continuous systems are average rather than maximum values, direct comparisons should be made with caution.

Consideration was given to the effects of permeability in in vitro rumens by Louw *et al.* (1949) who showed that semipermeable in vitro rumens permitted better cellulose digestion than the impermeable type. Gall & Glaws (1951) and Huhtanen, Saunders & Gall (1954) demonstrated that the microscopic appearance and motility of the microflora were more constant during prolonged in vitro incubation with a semipermeable system. El-Shazly, Dehority & Johnson (1960) compared three types of system directly, using VFA production, ammonia nitrogen (NH_3-N) production and cellulose digestion as criteria of microbial activity. The systems compared were all-glass, semipermeable and 'continuous-flow'. The continuous-flow

paratus did not, however, allow turnover of the actual in vitro culture but pro-ded for the diffusion of fermentation products and fresh substrate via a dialysing mbrane. The dialysing solution flowed continuously and contained a basal medium replenish the culture substrate. El-Shazly *et al.* (1960) concluded that there was le to choose, at least for short-term experiments of less than 30 h, between the stems.

Given the correct conditions, cultures of rumen micro-organisms can be main-ined for long periods in vitro, so that their microbiology and biochemistry reflect, to seful extent, the natural rumen. The principal attraction of an in vitro system is the gree of control and convenience it affords in practical research. A steady-state stem should exploit these properties to the full, as the usual variables are elimin-ed. The high degree of selectivity imposed by such a system will tend to produce s than perfect simulation of the natural rumen, but it lends itself to detailed icrobiological research since it offers a high degree of stability. For studies of a itritional or biochemical nature the continuous-flow, periodic-feed system, with ilk substrate retention in a nylon bag, appears to provide the greatest measure of alism in simulating the rumen.

A continuous, steady-state, in vitro rumen apparatus

A continuous, steady-state, in vitro rumen apparatus has been constructed at dinburgh in an attempt to meet all the criteria arising in the foregoing discussion. his equipment was not intended to simulate faithfully the natural rumen, but to ovide a highly controlled model system for comparative studies on the rumen icroflora.

The apparatus, shown diagrammatically in Fig. 1, has a glass culture vessel of o ml working volume with a stainless steel lid sealed by a silicone rubber gasket d fitted with ports for various services, electrodes and control elements. A mag-tic stirrer is fitted below the vessel. Complete mixing of culture is essential to eady-state working, but mixing must be as gentle as possible to avoid damage to the ost fragile of the rumen organisms. Hobson (1965) considered magnetic stirring to e too severe for rumen bacteria but Quinn (1962) used a magnetic stirrer at 120 rev./in in continuous pure culture of rumen ciliates.

Substrate delivery, antifoam addition and dialysis require accurate control, and a inexpensive means of providing this using miniature peristaltic pumps has been escribed (Ewart, 1973). Pumping substrate is a critical operation; continuity of ow and quality is essential. Satisfactory pumping is ensured by processing the ubstrate material in a double shear homogenizer (Alexander, 1969) to produce a ee-flowing slurry with a dry matter (DM) content adjusted to 4%. Foam formation the top of the culture results in irregular effluent removal since foam is generated rapidly as it leaves the vessel and, to counter the considerable tendency for in tro cultures to produce foam, a silicone antifoam agent is added at a controlled rate roughout the culture period.

Regenerated cellulose tubing is the usual dialysis membrane material used in

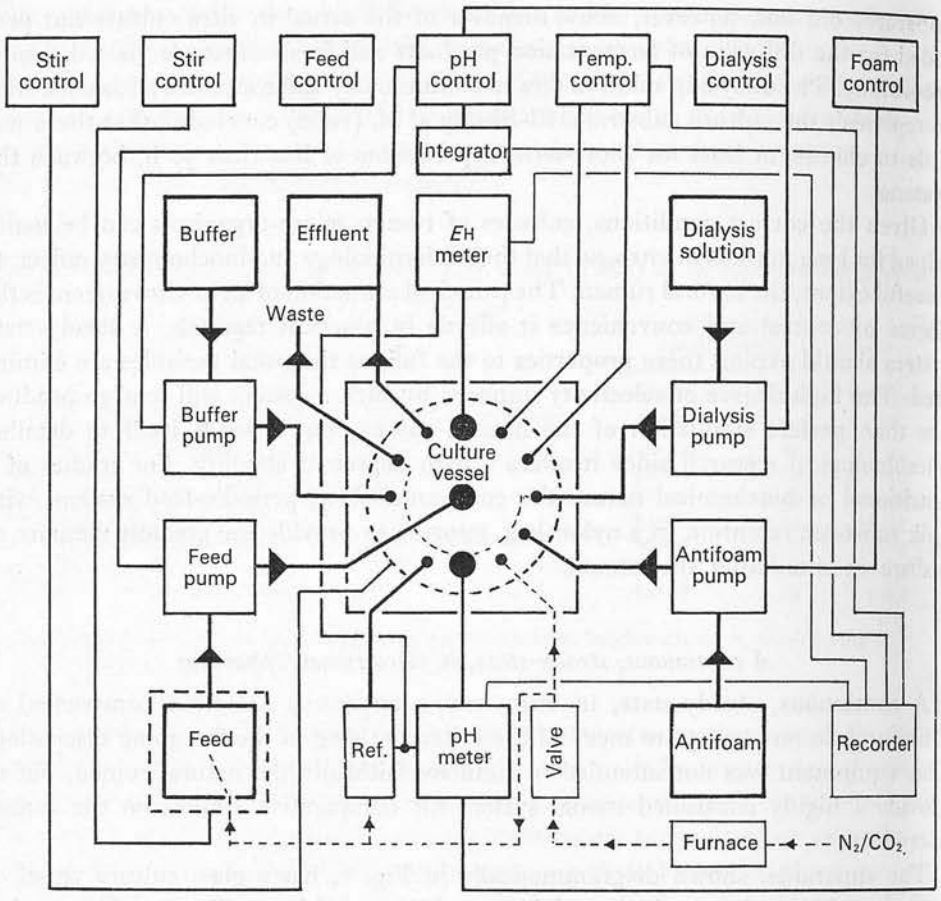

Fig. 1. Diagram of continuous, steady-state, in vitro rumen system: the 'Rumenstat'.

in vitro rumens, although it is suspect in an environment of intense cellulose digestion. Renal dialysis also uses regenerated cellulose membranes but, in response to permeability problems with this material, various synthetic copolymeric membranes have been devised (Muir, Courtney, Gray & Ritchie, 1971). These membranes were found to be immune to attack by the enzymes of the rumen microflora but the technology of their production has not yet reached a stage where they can be configured and sealed in the confines of a small culture vessel. In this apparatus, tubes of regenerated cellulose foil were used and found to be reliable, probably because the ordered molecular structure of this material precluded enzymic attack. The dialysis solution, a modification of the artificial saliva described by McDougall (1948), contained concentrations of salts calculated to be isotonic with those in the in vitro culture.

The pH and E_H of the culture are measured by conventional means with a common reference element serving the glass pH electrode and the platinum E_H electrode.

The pH-measuring circuit provides a signal for the pH control unit which, like all

the electronic circuitry, was specially built and makes use of linear integrated-cuit components. Artificial saliva, serving as the pH control agent, is delivered by a ristaltic pump. An integrator is connected to this control system and a recorder ce shows the rate of pH adjustment.

There are also control systems for the culture and feed-slurry stirrers, culture nperature and flow rate of oxygen-free gas. This gas (N_2–CO_2), which ensures aerobiosis by maintaining positive pressure in the apparatus, also provides ditional mixing in the culture vessel and transports liquid from the vessel via the fluent weir. Pressure-balancing pipes are necessary between the source of this pply, the various reservoirs and the reference electrode–liquid junction.

It is suggested that a steady-state, in vitro rumen apparatus of this type should be scribed as a 'Rumenstat'.

Use of Rumenstat in silage studies

The apparatus described has been used for studying the effects of silages on the men microflora. The work reported here is a series of trials in which silages were bjected to the same conditions in the Rumenstat and the steady-state cultures aracterized.

Table 3 describes the silages used: a control silage, a silage made using a 1:4 (w/w) ditive mixture of acetic acid and formic acid at 5·6 g/kg fresh grass and a silage ade with an acetic acid–formaldehyde mixture (1:1, w/w) at 9 g/kg fresh grass.

Table 3. *Analysis of silages used in the in vitro rumen system*

Silage	Dry matter (DM) (g/kg)	Nitrogen (g/kg DM)	Water-soluble carbohydrate (g/kg DM)	pH
Control	191	27·4	38	4·11
+Acetic acid–formic acid	198	27·7	96	4·12
+Acetic acid–formaldehyde	208	26·0	144	4·62

Microbiological techniques were based on those of Hungate (1966) and Blackburn Hobson (1962). Three groups of micro-organisms were enumerated by viable unts in media which selected for the ability to utilize cellulose, gelatin and starch specific substrates. NH_3-N concentration was determined by microdiffusion id VFA by gas-liquid chromatography.

An inoculum of a mixture of strained rumen liquor obtained from three fistulated ether sheep maintained on a constant diet of hay and concentrates was used at the art of each in vitro trial. The steady state was reached after approximately 72 h eration; this agrees with the findings of Slyter *et al.* (1964). Steady-state values of fferential viable counts, NH_3-N and VFA were obtained by calculating the mean analytical values on 3 successive days at 96, 120 and 144 h after inoculating the lture. As the values of NH_3-N were obtained on the day of sampling they were used a reference and if the coefficient of variation exceeded 5%, further samples were ken on subsequent days.

Samples were also taken for microbiological analysis from groups of three fistulated wether sheep on diets of the same silages, at 2 h post feeding, after a 14 d acclimatization period.

Steady-state analytical results are given in Table 4.

Table 4. *Steady-state analysis of in vitro rumen cultures maintained on supplemented silages*

Silage	Log (organisms/ml) Starch medium*	Gelatin medium*	Cellulose medium*	Ammonia nitrogen (mg/l)	Total volatile fatty acid production rate (mmol/l per h)
Control	6·63	6·58	5·77	76·35	3·8
+Acetic acid–formic acid	6·33	6·29	6·40	66·92	3·9
+Acetic acid–formaldehyde	6·46	5·38	5·51	28·45	3·6

*Counting media which selected for the ability to utilize starch, gelatin or cellulose.

More organisms were counted from the control silage culture than the treated silage cultures, on the starch medium ($P<0·05$). With the gelatin medium fewer organisms were counted in the instance of acetic acid–formaldehyde-treated silage ($P<0·05$). Counts on cellulose medium showed significant differences ($P<0·05$) among the three silages. These differences were also obtained with the fistulated sheep (Table 5).

Table 5. *Viable counts of rumen micro-organisms in fistulated wether sheep*

Silage	Log (organisms/ml) Starch medium*	Gelatin medium*	Cellulose medium*
Control	6·22	6·59	5·14
+Acetic acid–formic acid	5·78	6·54	6·57
+Acetic acid–formaldehyde	6·42	6·22	6·37

*Counting media which selected for the ability to utilize starch, gelatin or cellulose.

The microbiological results in vivo correlate well with those obtained in vitro in the instance of counts on gelatin and cellulose medium ($r=0·94$, $P<0·01$ and $r=0·85$, $P<0·05$). NH_3-N concentrations in vitro also correlate well with the counts on the gelatin medium ($r=0·94$, $P<0·01$).

Total VFA production rates were similar, suggesting that this is a characteristic of the culture conditions rather than the substrate. Metabolic energy production by the rumen micro-organisms is linked to growth rate and reflected in total VFA production. At a steady state, growth rate is fixed by the culture dilution rate, which was identical in each instance.

There appears to be considerable research potential in an apparatus of this type, especially because the culture conditions can be widely but accurately varied.

In addition, the stable and definable steady-state culture is an ideal means of determining directly the effects of various substrates on the rumen fermentation.

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Session 4

Paper 12

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This paper deals with the potential antimicrobial action of silage additives, in the rumen. Present knowledge of antimicrobial compounds which have been evaluated in the rumen is briefly discussed and in particular the possible modes of action of short and long chain fatty acids are considered.

An experiment is described in which "in vitro" ruminal cultures, maintained at steady-state on an additive-free silage diet in the "Rumenstat" apparatus, were infused with increasing concentrations of formaldehyde and the sodium salts of formic acid, acetic acid and propionic acid.

Results of these experiments show that the salts acted as inhibitors of VFA production and thereby brought about an apparent "energy deficit" for the microflora, resulting in an increase in ruminal NH_3-N concentration. The microbiological effects of these infusions were, however, limited; total numbers of organisms were maintained at high infusate concentrations.

Even with low concentrations of infused formaldehyde the effects on VFA production, NH_3-N concentration and microbial numbers were considerable suggesting a non-selective toxic action and differing from the catabolite repression effects apparently brought about by the organic acid salts.

The implications of these results in terms of both the modes of action of ruminal inhibitors and the additive treatment of silages, are discussed.